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TRABAJO FIN DE GRADO

DIFFERENTIATION OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS) INTO TUBULAR STRUCTURES RESEMBLING IMMATURE CAPILLARIES

Nombre:
Jorge Aranda Hernández
Titulación:
Bachelor in Biomedical Engineering - Tissue Engineering and Regenerative Medicine

Profesores:
Dr. Claudio Jorge Conti
Dra. Adela García Martín

Contents

Abstract

Angiogenesis is the mechanism of new vessel formation from pre-existing ones. It is a process of major relevance in tissue development and, more prominently, in pathological conditions. Kindler Syndrome, a rare disease affecting protein kindlin1, disrupts the dermo-epidermal junction resulting in skin blistering.

In order to interrogate the angiogenic effect of the Kindler Syndrome, bovine aortic endothelial cells and human umbilical vein endothelial cells were cultured in fibrin gel and Matrigel™ basement matrix. Qualitative analysis of the former indicated an enhanced angiogenic activity at basement membrane level induced by the Kindler genotype in human keratinocytes. This result was confirmed *a posteriori* by quantitative analysis with the Angiogenesis Analyzer plugin of ImageJ.

The quantitative results point to an increased release of mitogenic substances by keratinocytes lining the epidermis. This has the effect of enhancing angiogenesis in the dermal layer of the skin and is evidenced by the increased number of branches and branch length found under Kindler conditions, indicative of sprouting angiogenesis. Increased mesh area and number, along with a reduced mean mesh size found under Kindler conditions are indicative of an enhanced intussuspective angiogenic activity. The analysis of these five key parameters strongly suggest the proangiogenic influence of the Kindler genotype, expressed in epidermal keratinocytes, which affects vessel formation in the dermal layer of the skin.

State of the Art

The formation of new blood vessels from pre-existing ones or de-novo, phenomena known as angiogenesis and vasculogenesis respectively, are dynamic processes of great importance in the development of many conditions and biological processes. Embryonic development and growth, placental implantation in the endometrium and regeneration of cutaneous wounds are examples of common processes in which vessel formation is involved and plays a critical role [1,2]. In a similar way angiogenesis is key to the development of pathological conditions such as tumors, diabetic retinopathies, psoriasis or rheumatoid arthritis [3]. The recruitment of new blood vessels supports the progress of such pathologies, cancer being the most notorious case.

Tumor growth in its progression towards metastasis renders mass transport of nutrients through diffusion insufficient. Thus, cancerous tissue will generate a series of chemical substances of biological consequence which will result in angiogenesis. It is recognized that inflammation can contribute to proliferation, migration and survival of cancer cells by influencing the microenvironment around tumors [4,5], and oncogenes are known to build up an inflammatory pro-tumorigenic microenvironment [6].

Angiogenesis is the major mechanism of vessel formation in adults, being vasculogenesis more prominent during foetal development. It is therefore of great importance to understand the process of angiogenesis, how it is activated and how it progresses towards the formation of mature blood vessels from micro-capillaries. Moreover, it is important to understand how it is triggered and by what substances, in order to relate the formation of new capillaries to physiological conditions. This, as perhaps in the case of cancer, may give us the chance of palliating and even curing the given condition. Halting new blood vessel creation and disrupting the tumor microenvironment, was pointed as a convenient strategy for cancer treatment, so therapeutics have been used in combination with angiogenesis inhibitors to reduce cancer morbidity and mortality [7].

In order to visualize the formation of new blood vessels, an experimental technique is required which allows to recreate the physiological mechanism of blood vessel formation in a controlled environment. There is great a variety of

angiogenesis experimental setups, divided between *In Vitro*, *In Vivo* and *Ex Vivo* techniques. The first of the three consists of using cells extracted from a live system and cultured in laboratory glassware, with appropriate medium and extracellular matrix. The second major group represents the contrary to the previously described, as it involves an experiment occurring inside a living organism [8]. The third kind is a mix of the previous two, cells or tissues extracted from a live organism are manipulated *in vitro* and re-grafted into the organism for the experiment to happen inside. In general, biological investigation follows the following process: starting off with *in vitro* experiments and afterwards scaling-up the investigation by undergoing *in vivo* procedures. This scale-up not only implies an increase of the economic cost of the experiment, but also represents a scale-up in the complexity of the investigated system. As biological systems are extremely complex ones and parallel ongoing processes may produce noise on the results.

Different *in vitro* experiments are performed in order to tune-up an angiogenesis assay for the quantification of Kindler's Syndrome's pro-angiogenic activities. Four different experiments were performed, culturing two different types of endothelial cells and using two different types of extracellular matrices. The results are obtained by taking photographs of the cultured gels and by analyzing them in a qualitative manner first and, ultimately, quantitatively. Throughout this work, the advantages and disadvantages of each method are critically analyzed and the results obtained commented and compared with the existing literature.

The objective of the present research is, thus, to set-up an experimental procedure based on existing literature to study angiogenesis *in vitro* and the angiogenic influence of growth factor VEGF, inhibitor PEDF and conditioned media from keratinocytes. The angiogenic effect of Kindler's Syndrome at the dermo-epidermal junction level will also be interrogated and compared with the previous experimental groups, being the ultimate goal of the experiment the quantification of the different relative angiogenic activities.

Introduction

1. Cell Signaling Basis

The multicellular organism is, characteristically, highly organized into organs and tissues, where communication between different cellular types is key to maintaining controlled cell growth and differentiation. Cells may communicate between them following three distinct mechanisms:

A. Remote signaling through secreted molecules
B. Contact signaling through plasma-membrane bound molecules
C. Contact signaling via gap junctions

Types (A) and (B) are types of chemical signaling, in which a molecule from the emitter cell activates a receiver cell via a membrane-bound receptor. The difference, however, comes in the distance separating one cell from another. In case (A) the distance is large, so that the messenger must travel within the local tissue by diffusion or across tissues or organs traveling in the blood by convection, as would be the case of hormones in the endocrine system. Case (B) implies the direct contact of two cells to allow the interaction of their membrane-bound molecules. Gap junctions (C) are characteristic of epithelial tissues, tightly packed cells generally arranged in a monolayer. These 'gaps' in plasma-membranes allow epithelia to communicate with adjacent cells through chemical exchange between cytoplasms [9].

There are a series of worth mentioning features that are characteristic of cellular signal transducer systems:

A. Specificity: Only the signal molecule will bind to its target receptor, as any other molecule will not physically 'fit' into the receptor, thus, not interacting with its active site.
B. Amplification: To propagate a signal, enzymes activate other enzymes in a geometrically increasing manner. This amplifies the signal over time and space.
C. Desensitization and adaptation: When a receptor is activated, it cascades a negative feedback pathway which, in turn, either de-activates it or sequesters it into the cell's interior to elude over-exposure to the ligand.

D. Integration: The effect of two opposing or synergistic signals at receptor level result in an response integrating both stimuli.

1.1. Receptor Tyrosine Kinases and Mitogen Activated Protein Kinases

Receptor tyrosine kinases (RTK) are a family of plasma membrane bound proteins. They are characterized by having a ligand-specific binding domain in their extracellular domain and an enzyme active site in their intracellular domain. This cytoplasmic domain behaves as a tyrosine kinase, an enzyme family which phosphorylates tyrosine (Tyr) residues of specific target proteins.

The ligation of the receptor's extracellular domain to the ligand causes the intracellular domain to phosphorylate a series of proteins in a sequential manner (Figure 1). GRB2, without known enzymatic activity, serves as an adaptor molecule for the substrate of the RTK intracellular domain (IRS1-1 in the case of an insulin receptor, for example) and the molecule SOS, enlarging the receptor complex. SOS catalyzes the phosphorylation of the nucleotide guanosine bi-phosphate (GDP), bound to molecule RAS, into guanosine triphosphate (GTP). This activates RAS' kinase activity, allowing it to activate by phosphorylation the first of three protein kinases: Raf-1, MEK and ERK.

ERK pertains to the MAPK (Mitogen Activated Protein Kinases) family, known to be activated by MEK through phosphorylation. MEK is of the MAPKK (MAP kinase kinases) family and is, in turn, activated by Raf-1, of the family MAPKKK (MAP kinase kinase kinases). This sequential cascade, known as the MAPK cascade is an amplified intracellular signaling pathway that concludes with ERK entering the nuclei and phosphorylating transcription factors involved in the transcription and posterior translation processes. Thus, gene expression and protein production is the ultimate response to an stimulus that is transduced across the cytoplasm [10].

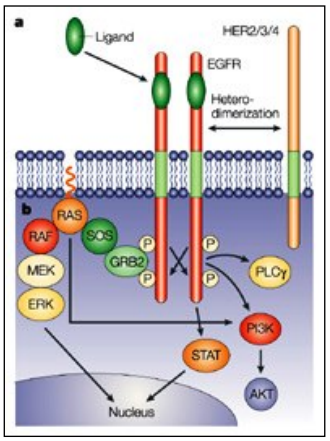


FIGURE 1: RECEPTOR TYROSINE KINASE

1.2. Integrins and Adhesion Receptors

Integrins are a family of plasma membrane protein receptors whose functions are to provide the cell with a means of interaction with the extracellular matrix (ECM) and other cells. Integrins are the major responsible for cell anchorage to the ECM but also allow for the bidirectional exchange of small molecules between the cell and the ECM. Structurally, integrins are dimers formed by an α and a β subunit, each with extracellular and cytoplasmic regions. There are 18 different α subunits encoded in the mammalian genome and only 8 β subunits, giving a wide variety of $\alpha^n\beta^m$ integrins. The dimerization the α and β subunits at plasma membrane level results in integrin activation.

Many extracellular ligands interact with integrins, like collagen, fibrinogen and fibronectin among others. In order for an integrin's extracellular domain to recognize a ligand, the latter must contain an amino acid sequence known as

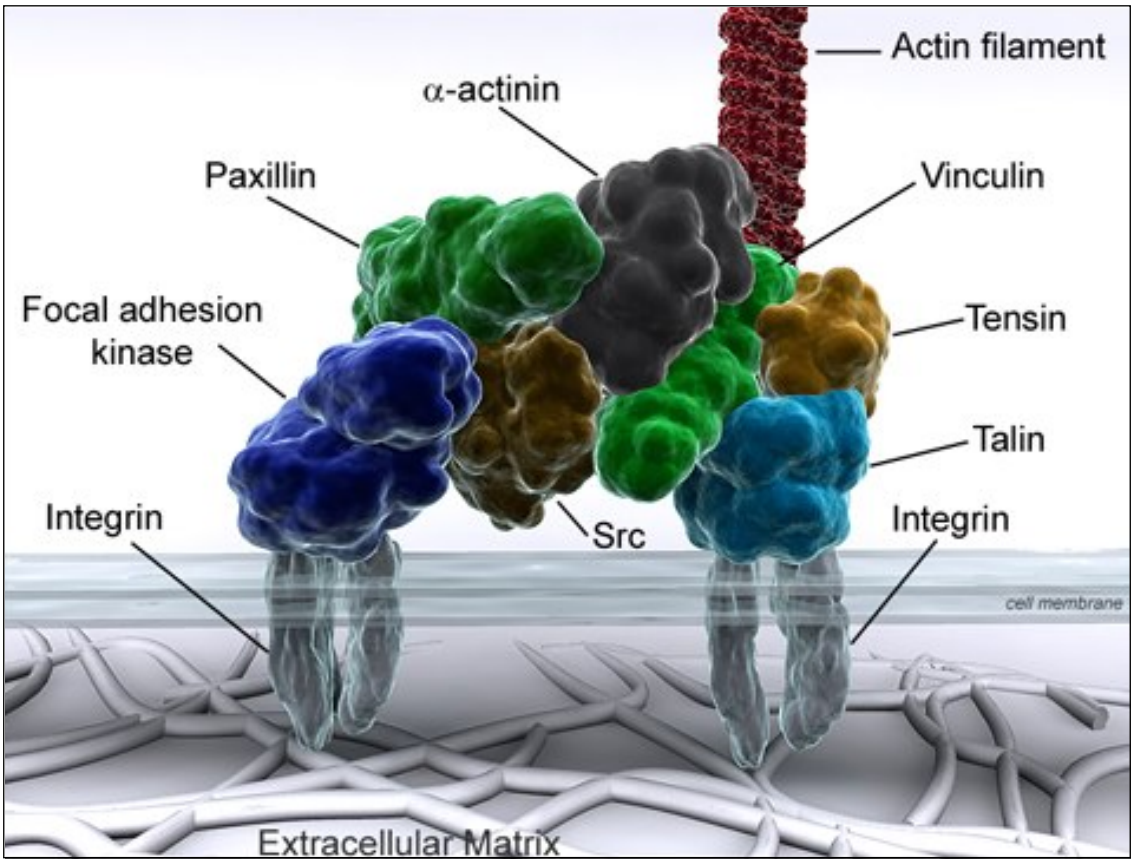


FIGURE 2: INTEGRINS AND THE (BASAL) FOCAL ADHESION COMPLEX

RGD, formed by arginine (Arg, R), glycine (Gly, G) and aspartic acid (Asp, D). The intracellular region is known to interact with a series of proteins: talin, α-actinin, vinculin and paxillin, among others (Figure 2). This way, integrins serve

as a bridge connecting the cell's cytoskeleton with the ECM, for structural and interaction purposes.

The dual association of integrins to both the ECM and cytoskeleton allow them to govern the mechanisms of cell shape, polarity, motility and differentiation in many cellular types as a response to extracellular stimuli. However, integrin conformation and adhesiveness can be altered from within the cell as a consequence of intracellular signaling., this way, the extracellular region of integrins may be conformationally activated to bind with the ECM, or committed to detach [10].

1.3. The Actin Cytoskeleton

The eukariotic cell's interior (cytoplasm) is filled with cytosol, a watery solution of ions and other small molecules, organelles with different functions and the cytoskeleton, responsible for the cell's motility, division and organelle rearrangement. The eukariotic cytoskeleton is comprised of three different polymers: actin filaments, intermediate filaments and microtubules. Cross-linking proteins rearrange actin filaments to form viscoelastic gels that connect transmembrane proteins and signaling complexes located at the extracellular matrix (EM) anchoring sites, i.e. focal adhesions. Talin is the main responsible protein for the linkage of actin filaments to β integrin tails [11].

A main characteristic of the cellular cytoskeleton is that it is an ever-changing structure, dynamic control of actin polymerization allows for cell motility and the rearrangement of its shape during, for example, adhesion in adherent cells. Actin filaments are continuously being elongated by the addition of actin monomers, but shrinkage may also occur if deemed necessary. If this growth mechanism is biased in space, the cell shall move in that direction as a response to the chemical bias. However poorly characterized are the molecular mechanisms that mediate the transduction of mechanical stimuli, signaling events may be initiated at the site of attachment of the cytoskeletal-polymers to sites of conformationally changing proteins [11].

2. Neovascularization and Angiogenesis

John Hunter, Scottish anatomist and surgeon, provided the first recorded scientific insights into the field of angiogenesis, suggesting an existing proportionality between vascularity and metabolic requirements, in both health and disease. His findings, published in 1794 [12] are as follows: "*In short, whenever Nature has considerable operations going on, and those are rapid, then we find the vascular system in a proportionable degree enlarged.*"

2.1.Blood Vessels

2.1.1.Cell Types and Origin:

The human body, not unlike any other pluricellular organism, is functionally divided into organ systems, each system consisting of different organs with different functions. Every organ is made of different cellular types, as cells are the minimal functional units of pluricellular beings. Thus, a same type of cells with the same function will be organized into a tissue within the organ. The way by which an organ, ultimately consisting of different highly specialized cell types, is produced in biology is called organogenesis and it occurs between the third and eighth weeks of the embryo's development in the mother's womb. Before organogenesis, however, the zygote (fertilized egg cell) undergoes an series of mitotic divisions without growth (cleavage) and differentiation, ending with the three germ layers: endoderm, mesoderm and ectoderm [13].

The cardiovascular system is the first organ system to develop in the embryo [14]. The luminal surface of the circulatory system, that in contact with blood, is a single layer of endothelial cells. These are derived from the mesoderm (Figure 3). Hemangioblasts differentiate from mesodermal stem cells and give rise

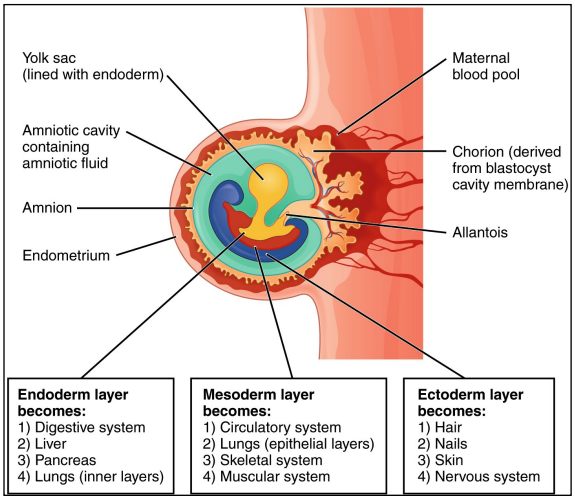


FIGURE 3: THE THREE GERMINAL LAYERS

to hematopoietic stem cells and angioblasts. Angioblasts are a cell type with potency to differentiate into endothelial cells which hasn't yet acquired all the characteristic markers of endothelial cells.

2.1.2. Vessel structure

Blood vessels are mainly divided into two categories, depending on the blood flow direction, i.e. to the heart or from the heart. The former class is commonly known as veins, whereas the second class are known as arteries. There are many differences in their structures, mainly driven by the pressure difference between veins and arteries. Though both have three different layers with different cellular types, the middle layer is thicker in arteries than it is in veins (Figure 4):

• *Tunica intima*: A single layer of simple stratified endothelial cells with a polysaccharide intercellular matrix. It is surrounded by a thin layer of connective tissue interlaced with circularly arranged elastic bands.

• *Tunica media* (thickest in arteries): circularly arranged elastin fibers and connective tissue. The tunica media in arteries is rich in vascular smooth muscle.

• *Tunica adventitia*: (thickest in veins) it is made of connective tissue. It also contains nerves and nutrient capillaries (vasa vasorum) in large blood vessels.

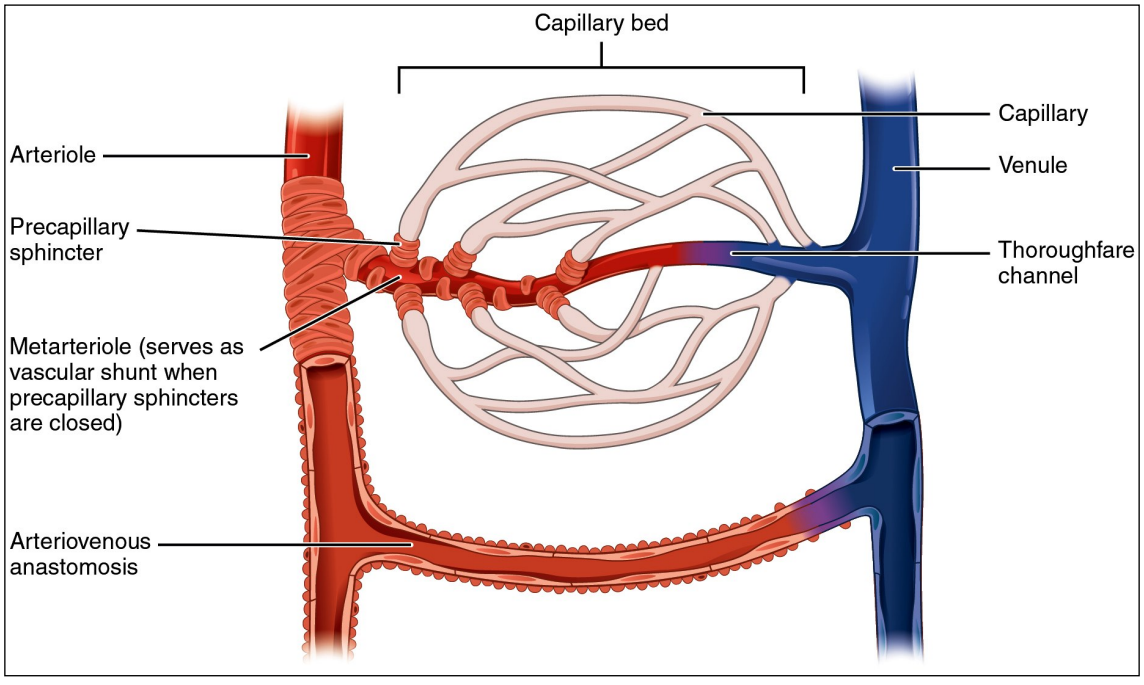


FIGURE 4.B: THE CAPILLARY BED AND ANASTOMOSIS

Capillary and microcapillary vessels are characterized by their small cross-section and consist of little more than a layer of endothelium and occasional connective tissue. These microvessels, measuring around 5μm to 10μm in diameter, connect arterioles and venues (see Figure 4.A and 4.B) and they allow for exchange of nutrients and waste chemical substances between blood and the tissues by simple diffusion, due to the thinness of their walls [15]. When blood vessels connect to form a region of diffuse vascular supply it is called an anastomosis, which provides alternative routes for blood to flow in the case of blockages.

2.2. Mechanisms of Blood Vessel Formation

2.2.1.Vasculogenesis

Vasculogenesis is the formation of blood vessels from angioblasts de novo. This process includes differentiation of mesodermal stem cells into angioblasts and the growth factor directed migration of angioblasts to form blood islands, where angioblasts give rise to endothelial cells [13-18]. Note that the blood islands and vessels *outside* of the embryo are initially the sole source of blood cells and plasma, beginning 3 weeks after fertilization [19].

The dynamic process of vasculogenesis involves cell–cell and cell–extracellular matrix (ECM) interactions directed spatially and temporally by growth factors and morphogens [20-23], most prominently during fetal development than in adult life.

2.2.2. Angiogenesis

Angiogenesis occurs both in embryos and during adulthood and, as previously stated, it is the physiological process through which new blood vessels are formed from pre-existing vessels (Figure 5).

a. Sprouting Angiogenesis

It occurs through a series of well-defined stages. Firstly, angiogenic growth factors (biochemical signals) activate membrane-bound receptors on endothelial cells forming pre-existing blood vessels. Then, activated endothelial cells start releasing proteinases for extracellular matrix degradation, to allow endothelial cells to escape from the original vessel wall. Proliferation into the

surrounding matrix and formation of solid sprouts connecting neighboring vessels always happens toward the source of the angiogenic stimulus.

Hypoxia, namely the lack of required levels of oxygen, is a main reason for the sprouting of new blood vessels in order to increase the blood supply. Parenchymal cells (myocytes, hepatocytes, neurons…) respond to hypoxia environment by secreting a key proangiogenic growth factor called Vascular Endothelial Growth Factor (VEGF). An endothelial tip cell guides the developing capillary sprout through the ECM toward an angiogenic stimulus such as VEGF-A [24], which will be of great relevance in this study.

Filopodia are long and thin cellular appendages located on tip cells which secrete large amounts of proteolytic enzymes and are heavily endowed with VEGF-A receptors (VEGFR1 and VEGFR2) allowing them to align with the VEGF-A gradient. Contraction of actin filaments within the filopodia pull the tip cell along toward the VEGF-A stimulus [25].

b. Intussusceptive Angiogenesis

Intussusceptive angiogenesis, or splitting angiogenesis, is caused by the vessel wall extending into the lumen producing a single vessel to split into two (Figure 5). It is faster and more efficient than sprouting angiogenesis as it only requires reorganization of pre-existing endothelial cells and, thus, doesn't rely on immediate endothelial proliferation or migration [26].

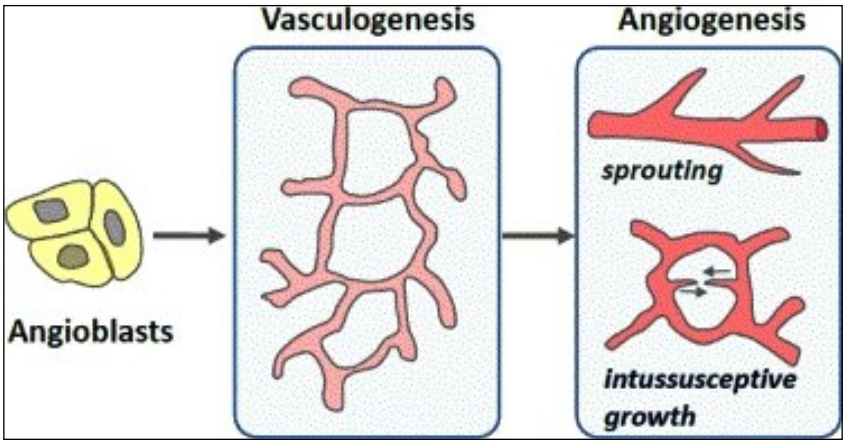


FIGURE 5: VASCULOGENESIS AND ANGIOGENESIS

2.3. Chemical Regulation of Vascularization

Angiogenesis is, as previously stated, a dynamic process. The purpose of vasculature in the vast majority of tissues (i.e. heart, brain, skeletal muscle …) is to convey nutrients and waste products to and from the given tissue,

respectively. Blood vessels have thus a nutritive function and, as Aristotle said: 'form follows function' [27]. Therefore, the regulation of endothelial cell migration and vasculature formation will depend upon the metabolic activity of the target tissue. However, metabolite-dependent formation of blood vessels may not be the only mechanism of blood vessel formation. Living cells have been discovered to process mechanical stimuli by mechanotransduction, its conversion to chemical signals [28].

August Krogh (1884-1949) designed a method for the structural characterization of vascular networks, it consisted on counting the number of capillaries per unit area of muscle cross section: the *capillary density.* Using this method, he concluded that the density of capillaries in the skeletal muscles is proportional to the basal metabolic rate of the animal [29]. His results where complemented later in time, by correlating the capillary length density with the mitochondrial volume density (Figure 6) as more rigorous magnitudes to quantify capillarity and metabolic rate (note that mitochondria are responsible for energy production in the cell) [30].

2.3.1.Oxygen

As previously stated, oxygen is the critical chemical driver of blood vessel formation, due to its high metabolic relevance. Though not the only substance involved in cellular metabolism, the relatively small capacity for its

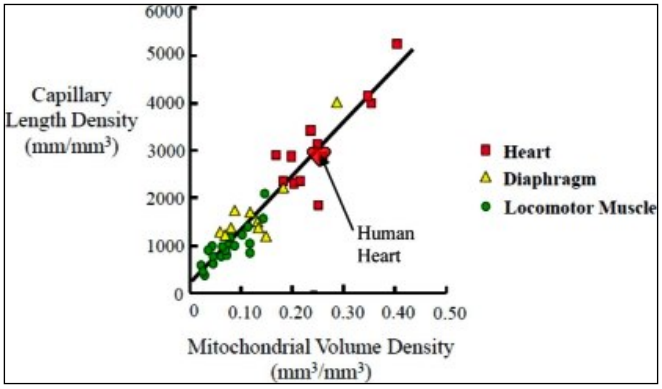


FIGURE 6: CAPILLARITY VS. METABOLIC RATE

storage in the body when compared to glucose, fats, etc. makes it critical in dictating vascularization. Therefore, hypoxic (under oxygenated) and/or ischemic (under irrigated) tissues will secrete many proangiogenic factors like VEGF-A [31] and FGF-2 [32] among many others, which may up-regulate

reciprocally. An example of this is hypoxia-inducible factor 1 (HIF-1), a major up-regulator of VEGF-A [33] produced by tissues in hypoxia conditions.

2.3.2. Vascular Endothelial Growth Factor (VEGF)

The VEGF family (VEGF-A, B, C, D, F) is a growth factor subfamily of the platelet-derived growth factor family. All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (VEGFRs) bound on the cell surface, causing them to dimerize and become activated through phosphorylation. The VEGF receptors (Figure 7) have an extracellular portion consisting of seven immunoglobulin-like domains, a single transmembrane spanning region, and an intracellular portion containing a split tyrosine-kinase domain [34].

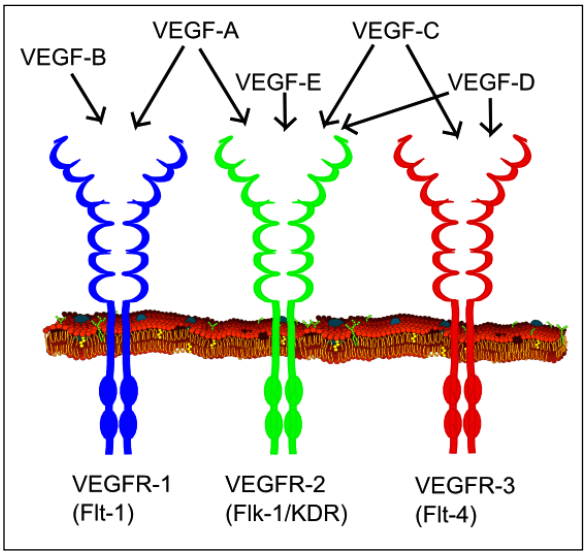


FIGURE 7: DIAGRAM OF VEGF TRANSMEMBRANE RECEPTORS

Rho GTPases are essential for vascular endothelial growth factor-mediated angiogenesis [35]. RhoA pathway (Figure 8) critically regulates angiogenesis driven by VEGF and constitutively-active RhoA (RAS homolog A family) can complement VEGF in order to increase its vascularizing effects. It has also been shown that VEGF induces RhoA activity and recruitment in the plasma membrane. VEGF stimulation of VEGF-receptors results in the formation of stress fibers (FS), a prominent family of F-Actins, which are linked to the plasma membrane and focal adhesions and have been related by many studies to endothelial cell formation in unfavorable conditions [36].

2.3.3. Wound Conditions: Fibroblast Growth Factor (FGF) and Mitogen Related Protein 3 (MRP3)

FGFs are promiscuous growth factor proteins. They are most commonly mitogens but also have regulatory, morphological, and endocrine effects; most notoriously angiogenesis, keratinocyte organization and wound healing

processes in mature systems [37]. One important function of FGF1 and FGF2 is the promotion of endothelial cell proliferation and their organization into tube-like structures.

FGF1 and FGF2 are more potent angiogenic factors than vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF). FGF1 and FGF2 stimulate angiogenesis and the proliferation of fibroblasts that give rise to granulation tissue, which fills up a wound space/cavity early in the wound-healing process. FGF7 and FGF10 (also known as Keratinocyte Growth Factors KGF and KGF2, respectively) stimulate the repair of injured skin and mucosal tissues by stimulating the proliferation, migration and differentiation of epithelial cells, and they have direct chemotactic (chemically mediated migration) effects key to tissue remodeling [38].

MRPs regulate endothelial cell migration and proliferation through interactions with cell-surface receptors, like the insulin-like growth factor 2 (IGF2)/mannose 6- phosphate receptor, MRP/proliferin receptor, or other unidentified receptors [39].

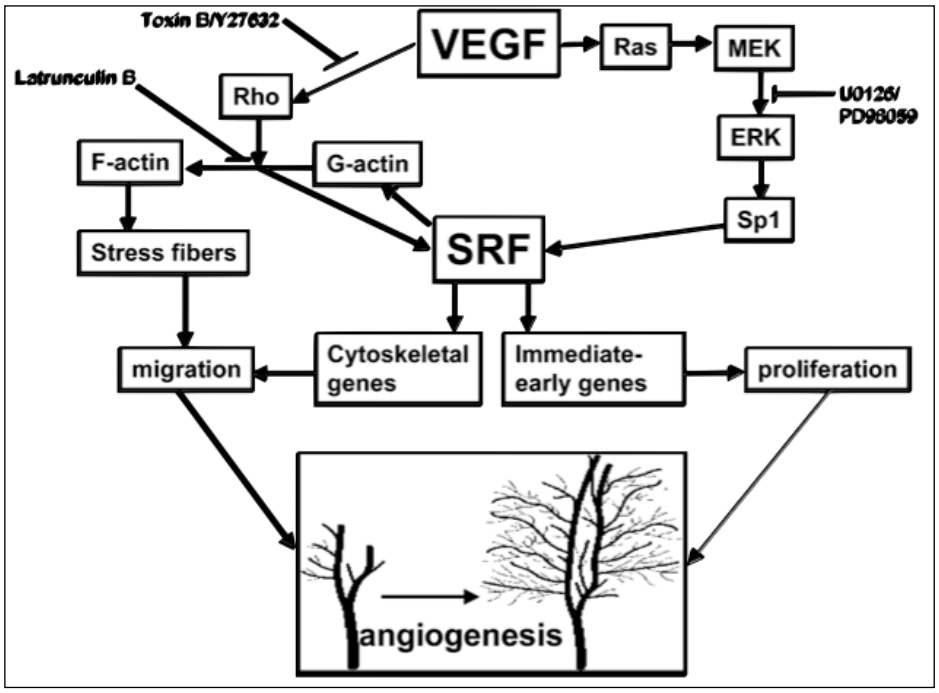


FIGURE 8: RHO SIGNALING PATHWAY

3. Human Skin and The Kindler Syndrome

3.1. Human Skin

Skin is, in humans the largest of all organs, with a total weight of 3-5kg and a surface area of 1.5-2m^2 in the average human [40]. Skin plays many very important roles like temperature regulation, sensation, hydration preservation and protection from pathogens. Its structure may be divided into two main layers: the epidermis and the dermis (Figure 9), each with a characteristic function and morphological structure; and separated from each other by the basal lamina.

3.1.1.The Epidermis

The epidermis is the skin's outermost layer. It's formed by stratified epithelial cells and has the purpose of acting as a physical barrier against many mechanical, chemical, thermal and radiative insults. Though only ~0.1mm thick, the epidermis consists of five different strata:

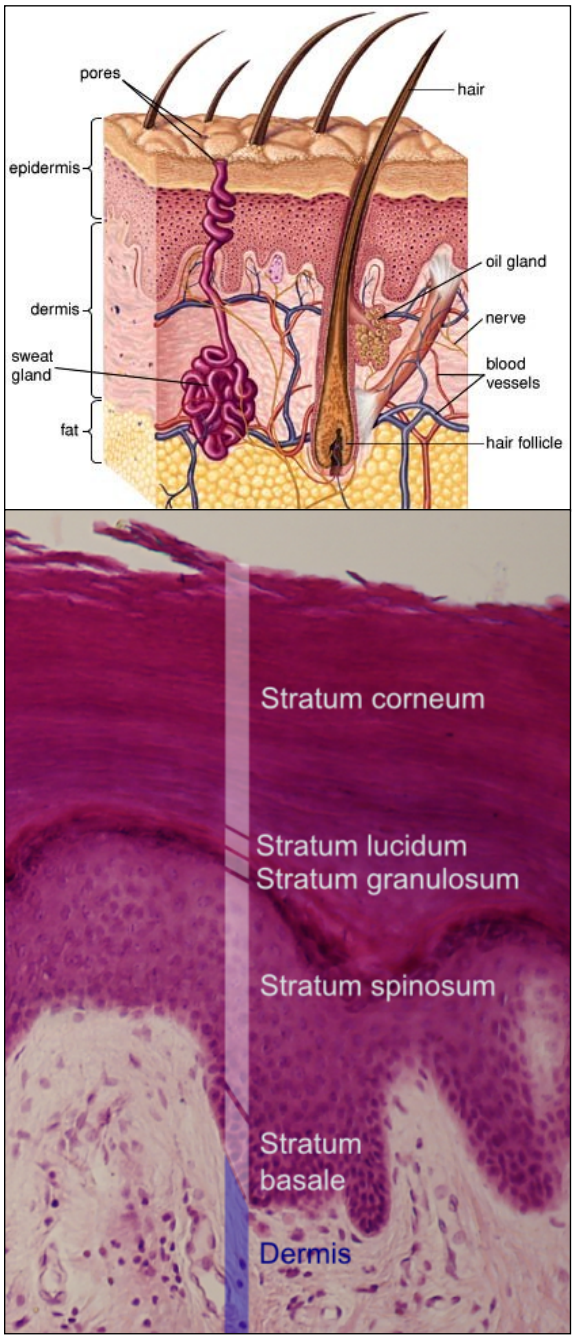
i. Basal/Germinal layer (stratum basale)
ii. Prickle cell layer (s. spinosum)
iii. Granular layer (s. granulosum)
iv. Translucid layer (s. lucidum)
v. Horny layer (s. corneum)

These strata follow a differentiation gradient perpendicular to the skin surface. The basal cells, closest to the dermis, are immature and constantly undergoing mitosis. With maturation, cells migrate toward the skin surface and once in the granular layer start producing keratin, which fills them up before senescence when reaching the horny layer [41]. Thus, the main cellular component of the epidermis are keratinocytes [42], i.e. keratinized epithelium and a minority of miscellaneous cells like melanocytes, Merkels or Langerhans cells, which are out of the scope of the present study.

Worth mentioning of the epidermis is that it is devoid of nervous tissue and blood irrigation. Therefore, the distal strata must rely on oxygen diffusion from the surrounding air for nourishment. This is a main driver of surface keratinocytes death and of the aforementioned differentiation gradient.

3.1.2.The Dermis

The dermis is the innermost layer of skin and is formed by two distinct strata, the papillary and reticular derma. The papillary dermis, which lies nearest to the epidermis is characterized by a series of projections, dermal papillae (Figures 9-10), which penetrate into the epidermis and account for the existence of fingerprints [43]. This layer's ECM is mainly comprised of loosely-packed collagen fibers and nervous loops, some termini of which end in the epidermis.



FIGURES 9-10: SCHEMATIC DIAGRAM OF THE SKIN (9) AND H&E STAINED HISTOLOGICAL CUT (10)

The main bulk of the dermis is given by the reticular layer, made of sturdier collagen fibers disposed in a reticulately interlaced and closely-packed manner. Besides collagen, whose main function is to enhance mechanical strength, elastin fibers can also be found in the reticular layer. The role of elastin fibers, much longer and amorphously conformed than collagen, is to give elastic properties to the dermis. The combination of mechanical strength and elasticity conferred by collagen and elastin make it an extremely robust tissue [41].

The main cellular component of the dermis are fibroblasts [42], which are not epithelial cells, but mesenchymal cells which originate from the mesodermal layer of the embryo, which produces most connective tissues. The dermis

contains a number of skin appendages (hair follicles, sebaceous glands) and, most importantly, a high amount of blood vessels. The high degree of irrigation and sturdiness the dermis are vital for the dermal support of the epidermis, as the epidermis relies on the dermal papillae for metabolic support [41] and anchorage.

3.1.3.The basal lamina

Stratified epithelial cells, like those forming the epidermis present a high degree of functional polarization between their basal and apical surfaces, that is, the surfaces in contact with the connective tissue and the opposite one, which lines the lumen of cavities. At the interphase between epithelial and connective tissues, independently of the organ system, a sheet of extracellular matrix is fund, called the basal lamina (BL) or basement membrane .The macromolecular components of the BL are secreted by epithelial basal poles and organized into a complex, interlaced, three-dimensional matrix. Including:

- Laminin: Glycoprotein (protein presenting carbohydrate cues) assembled into a lace-like structure onto which integrins bind for cell adhesion.
- Type-VII Collagen: A mesh of colagen endows the BL with mechanical resistance to tension and shear.
- Entactin and nidogen: Adhesive proteoglycans (hydrocarbon with peptide cues) with the function of holding together laminin and type-VII collagen.
- Perlecan: Proteoglycan with a similar function to that of entactin and nidogen.

Though morphologically differentiated, the BL becomes a diffuser meshwork towards the dermis, becoming the reticular lamina (RL). The RL is rich in type-III collagen, which binds to the basal lamina through type-VII collagen fibrils (Figure 11).

BM functions are diverse, including cell adhesion and communication. Epithelial cells to adhere to the BM adjacently to the connective tissue on which they rely for survival [44]. Epithelial cells may attach to the BM through focal adhesions (involving the actin cytoskeleton) or hemidesmosomes (through intermediate filaments) [45]. The BM, thus, mediates many cell-to-cell interactions which involve epithelial migration and serves as a growth factor

reservoir and filter for growth factors and mitogens coming from the connective tissue layers [44].

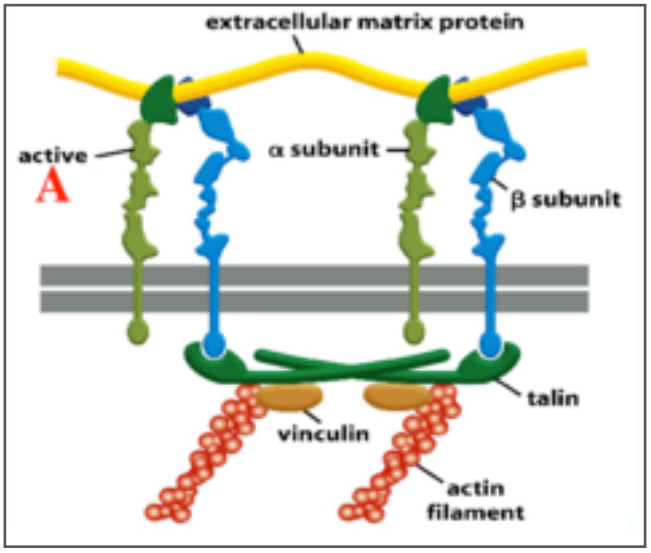


FIGURE 11: MACROMOLECULAR STRUCTURE OF THE FOCAL ADHESION

3.2.Kindler's Syndrome

Kindler Syndrome (KS) is a rare condition characterized by blistering, increased photosensitivity, patchy discoloration and widespread skin breakdown, symptoms collectively known as poikiloderma. Kindler syndrome is one of four types of Epidermolysis Bullosa, which affects ~1/53000 people in the world [46].

Caused by mutations in the FERMT1 gene (also called the KIND1 gene), KS is inherited in an autosomal recessive manner [47]. Gene KIND1 encodes protein Kindlin 1, from the kindlin protein family (Figure 11), thought to be involved in the anchorage of the actin cytoskeleton to integrin-associated signaling platforms [48]. Thus, KS is characterized by impaired actin cytoskeleton-extracellular matrix interactions [49].

FIGURE 12: KINDLER SYNDROME PATIENT

Kindlin 1 is a complex protein and many of its functions remain unknown. In silico analysis shows a FERM domain interupted by a Pleckstrin Homology (PH) domain. FERM proteins provide a regulated linkage from filamentous actin in the cortex to membrane proteins on the surface of cells. Their conformation and activity are regulated

by a combination of phospholipid binding and phosphorylation and seem to play a crucial role in the cellular cytoskeletal response to Rho path- way activation [50]. The PH domain has a strong specificity for plasma protein binding [51] (see figure 13).

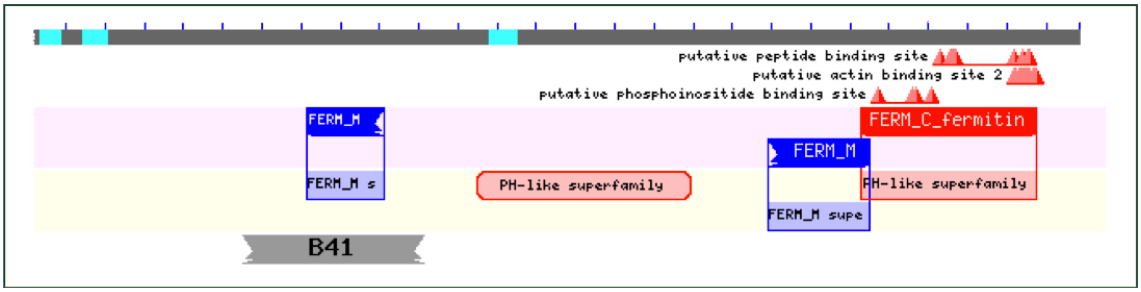


FIGURE 13: PROTEIN KINDLIN1 CONSERVED DOMAINS.

Herz et al. in their work *Kindlin-1 Is a Phosphoprotein Involved in Regulation of Polarity, Proliferation, and Motility of Epidermal Keratinocytes*, were able of co-localizing GFP-tagged kindlin 1 along with vinculin, actin, α3 and ß1 integrins. In fact, α3β1 integrin deficiency in keratinocytes leads to reduced mRNA and protein expression of the proangiogenic factor mitogen-regulated protein 3 (MRP3) [52]. Mutations in the kindlin1 protein are, therefore, bound to interfere with the actin-cytoskeleton adhesion functions and signal transduction mechanisms of the focal adhesions, causing a faulty intracellular signals to mediate the expression of genes which would be expressed under normal conditions. The purpose of the present study is to determine whereas dysfunctional focal adhesions, due to the Kindler Syndrome, result in an increased release of angiogenic substances by keratinocytes of the epidermis.

Materials and Methods

The conditioned media and cells used in the three experiments were provided from researchers working at CIEMAT (*Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas*). They were obtained from healthy wild-type human keratinocytes and Kindler Syndrome affected keratinocytes. For the first set of experiments, where BAEC where used, conditioned media from VEGF over-expressing keratinocytes was used, however, human recombinant VEGF is used for the last two.

1. Bovine Aortic Endothelial Cells (BAECs)

Bovine Aortic Endothelial cells (BAEC) provide an excellent model system to study all aspects of cardiovascular function and disease, such as critical signaling pathways and mechanisms relevant to proper function of endothelia, including angiogenesis.

BAECs obtained from CIEMAT were grown until confluence in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich), supplemented with 5mM L-glutamine, 10% FBS and an antibiotic mixture of 100 units/ml penicillin and 100 µg/ml streptomycin. P100 dishes coated with 0,5% gelatin were used for expanding the BAEC. 3 ml of 0,5% gelatin were added per dish and exposed to UV light for 30 minutes. Then the excess of gelatin is removed, being now the dishes prepared for the seeding.

Purity of the BAEC preparation was determined by cell morphology using inverse phase-contrast microscopy. Passages were performed after PBS wash (phosphate buffered saline) and detached from the gelatin plate using 1% trypsin. Both solutions were provided at our laboratory. Only cells passaged less than 10 times were used for experiments.

1.1. Culture in Fibrin Gel

The purpose of this experiment was to compare the angiogenic effects of Kindler-conditioned medium with simple cell culture medium, as a negative control and VEGF-conditoned medium (as a positive control).

On each of six wells are deposited 250μl of BAEC suspension in DMEM (10% FBS) with a total of $3{,}5 \cdot 10^5$ cells. For the preparation of the fibrin gel, fibrinogen is added, followed by thrombin from human plasma in solution with of $CaCl_2$ (50mM) to a total volume of 1ml (Figure 14). Photographs will be taken at 0, 24 and 48h.

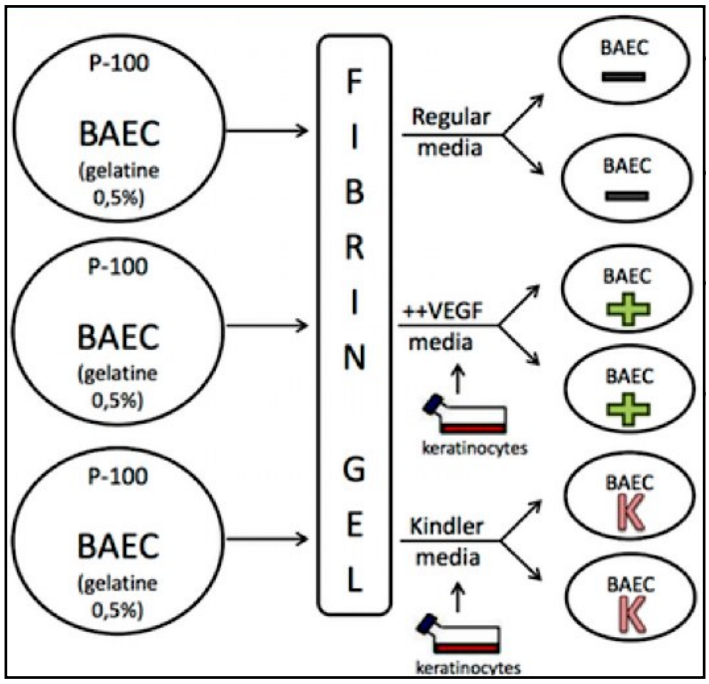


FIGURE 14: SCHEME OF THE PROCEDURE FOLLOWED FOR THE FIBRIN GEL EXPERIMENT

1.2.Culture in Matrigel™

The experiment was performed at two different conditions of cell density and Matrigel™ concentration. The purpose of this experiment was to addition conditioned media from healthy (wild type) human keratinocytes in order to compare the effect of kindler-conditioned angiogenesis to that influenced by normal keratinocytes.

Matrigel™ Basement Membrane Matrix was obtained from BD Biosciences®, and kept on ice so polymerization did not take place. Hence the two dilutions could be prepared, first at a proportion of 1:1 (Matrigel:DMEM without FBS) and second at 1:2.

Wells of a 96-well plate were then coated following the BD Biosciences® procedure to obtain thick Matrigel™, dispensing 65µl of the 1:1 solution per well and in the second case 65µl of the 1:2 solution per well, allowing it to gel at room temperature for 30 minutes. After polymerization was completed, BAEC

were seeded on top of the gels. For the first mixture (1:1) $1{,}5 \cdot 10^5$ cells/well were suspended in 100µl of DMEM and the remaining 185µl of volume was filled with conditioned medium. In the second case (1:2) a smaller amount of $2 \cdot 10^4$ cells/well were used but this time they were re-suspended only with regular DMEM in a total volume of 300 µl. After one hour, DMEM was removed and replaced by 300µl of corresponding conditioned medium. Bright-field images, using a 10× objective, were taken using an inverted phase-contrast microscope one hour after the medium was changed, plus 20 and 48 hours after.

• 100µl DMEM (10% FBS) • 1,5•10 • 65µl Thick Matrigel	• 100µl HK-WT cond. med. • 1,5•10 • 65µl Thick Matrigel	• 100µl VEGF cond. med. • 1,5•10 • 65µl Thick Matrigel	• 100µl DMEM (10% FBS) • 2•10 • 65µl Thick Matrigel	• 100µl HK-WT cond. med. • 2•10 • 65µl Thick Matrigel	• 100µl VEGF cond. med. • 2•10 • 65µl Thick Matrigel
• 100µl DMEM (10% FBS) • 1,5•10 • 65µl Thick Matrigel	• 100µl HK-WT cond. med. • 1,5•10 • 65µl Thick Matrigel	• 100µl VEGF cond. med. • 1,5•10 • 65µl Thick Matrigel	• 100µl DMEM (10% FBS) • 2•10 • 65µl Thick Matrigel	• 100µl HK-WT cond. med. • 2•10 • 65µl Thick Matrigel	• 100µl VEGF cond. med. • 2•10 • 65µl Thick Matrigel
• 100µl DMEM (10% FBS) • 1,5•10 • 65µl Thick Matrigel	• 100µl HK-WT cond. med. • 1,5•10 • 65µl Thick Matrigel	• 100µl VEGF cond. med. • 1,5•10 • 65µl Thick Matrigel	• 100µl DMEM (10% FBS) • 2•10 • 65µl Thick Matrigel	• 100µl HK-WT cond. med. • 2•10 • 65µl Thick Matrigel	• 100µl VEGF cond. med. • 2•10 • 65µl Thick Matrigel

TABLE 1: SCHEMATIC REPRESENTATION OF THE 96-WELL PLATE OF EXPERIMENT

2. Human Umbilical Vein Endothelial Cells (HUVECs)

Human Umbilical Vein Endothelial Cells (HUVEC) provide a classic model system to study many aspects of endothelial function and disease, such as normal, abnormal and tumor-associated angiogenesis, oxidative stress, hypoxia and inflammation related pathways in endothelia under normal and pathological conditions. Being of human origin, as are the stimulants and conditioned media in use, the use of HUVECs was introduced as an improvement of the previous two experiments. Besides, the introduction of human recombinant VEGF was meant to enhance vascularization in the positive control in order to have a more robust comparison. For this same

purpose, PEDF-conditioned medium from human keratinocytes (again, courtesy of CIEMAT) shall be used as an angiogenesis and vasculogenesis inhibitor in order to enhance the negative controls.

Triplicate wells of a pre-chilled 96-well plate are covered with 50μl of undiluted Matrigel®, and left to polymerize at 37ºC during 10 minutes. A HUVEC suspension with a total of $5{,}4 \cdot 10^5$ cells is prepared in Medium 199 (Sigma Aldrich) supplemented with 5% FBS and seeded on top of the Matrigel™ at a density of $3 \cdot 10^4$ cells per well. The plate is left to incubate at 37ºC for one hour in order for the HUVEC to adhere. The different media are prepared: DMEM (2% FBS), PEDF conditioned-media from human keratinocytes, M199 (10% FBS) and M199 (10% FBS) with 100ng/ml VEGF.

After 1h, when the HUVEC had adhered, the M199 media is removed from each well and replaced with 100μl of each corresponding medium, in a triplicate manner (Table 2). Photographs will be taken 6 and 24h from this moment. Quantitative analysis of the photographs is performed using the Angiogenesis Analyzer Plugin of the NIH ImageJ software, developed and presented by Gilles Carpentier on the *ImageJ Developer and User Conference* (2012). Preprocessing of the images, namely histogram equalization and band-pass spatial frequency filtering, is performed prior to the analysis.

• 100μl **DMEM** 5% FBS • 3x10^4 HUVEC • 50μl Matrigel®	• 100 μl **PEDF** • 3x10^4 HUVEC • 50μl Matrigel®	• 100μl **M199** • 5% FBS • 3x10^4 HUVEC • 50μl Matrigel®	• 90μl M199 5% FBS • 10μl **VEGF** (1μg/ml) • 3x10^4 HUVEC 50μl Matrigel®	• 100 μl HK-**WT** • 3x10^4 HUVEC • 50μl Matrigel®	• 100 μl HK-**Kindler** • 3x10^4 HUVEC • 50μl Matrigel®
• 100μl **DMEM** 5% FBS • 3x10^4 HUVEC • 50μl Matrigel®	• 100 μl **PEDF** • 3x10^4 HUVEC • 50μl Matrigel®	• 100μl **M199** • 5% FBS • 3x10^4 HUVEC • 50μl Matrigel®	• 90μl M199 5% FBS • 10μl **VEGF** (1μg/ml) • 3x10^4 HUVEC 50μl Matrigel®	• 100 μl HK-**WT** • 3x10^4 HUVEC • 50μl Matrigel®	• 100 μl HK-**Kindler** • 3x10^4 HUVEC • 50μl Matrigel®
• 100μl **DMEM** 5% FBS • 3x10^4 HUVEC • 50μl Matrigel®	• 100 μl **PEDF** • 3x10^4 HUVEC • 50μl Matrigel®	• 100μl **M199** • 5% FBS • 3x10^4 HUVEC • 50μl Matrigel®	• 90μl M199 5% FBS • 10μl **VEGF** (1μg/ml) • 3x10^4 HUVEC 50μl Matrigel®	• 100 μl HK-**WT** • 3x10^4 HUVEC • 50μl Matrigel®	• 100 μl HK-**Kindler** • 3x10^4 HUVEC • 50μl Matrigel®

TABLE 2: SCHEMATIC REPRESENTATION OF THE 96-WELL PLATE OF THE HUVEC EXPERIMENT

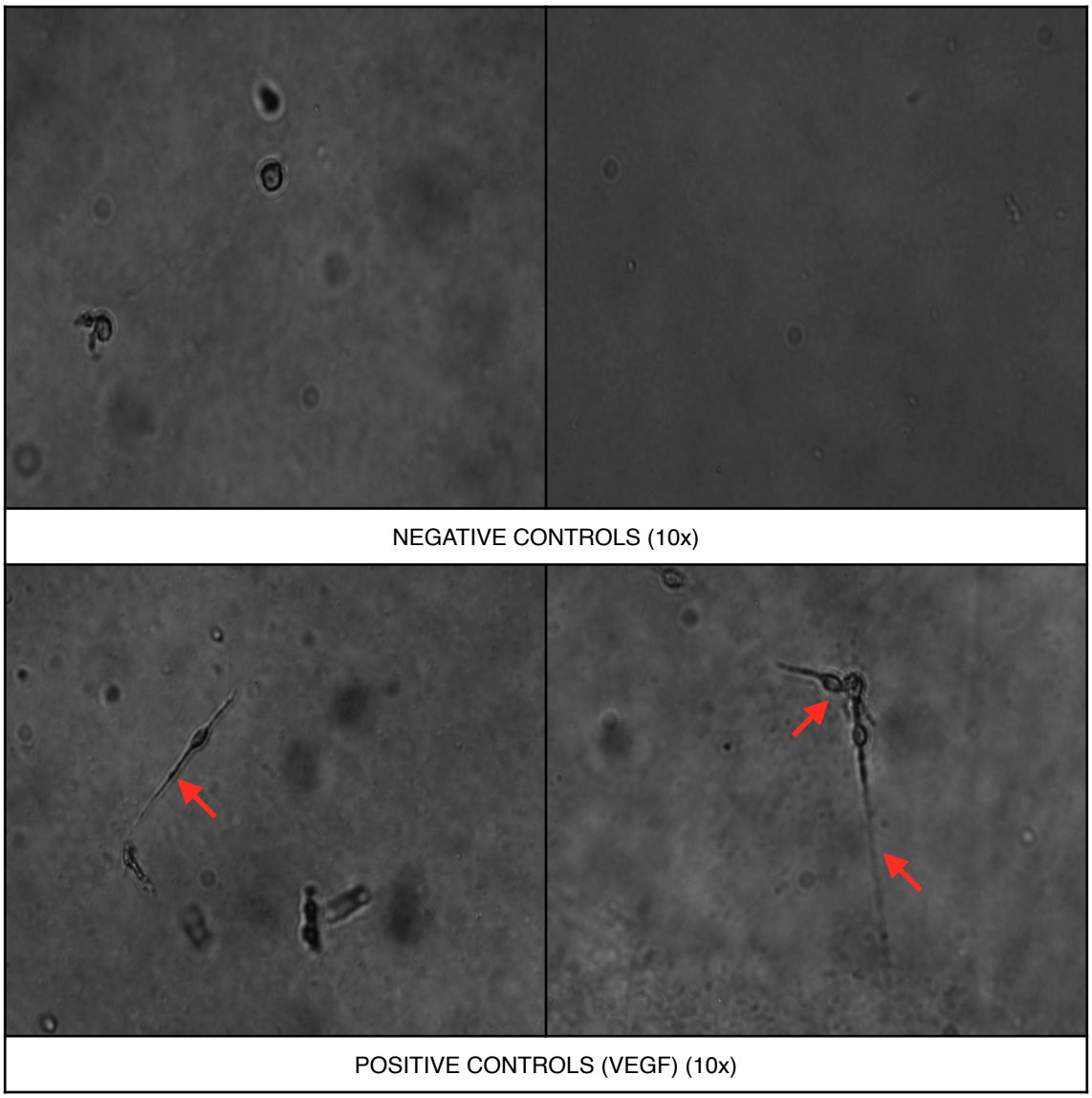
Results

1. BAEC culture on fibrin gel

Results after 24h:

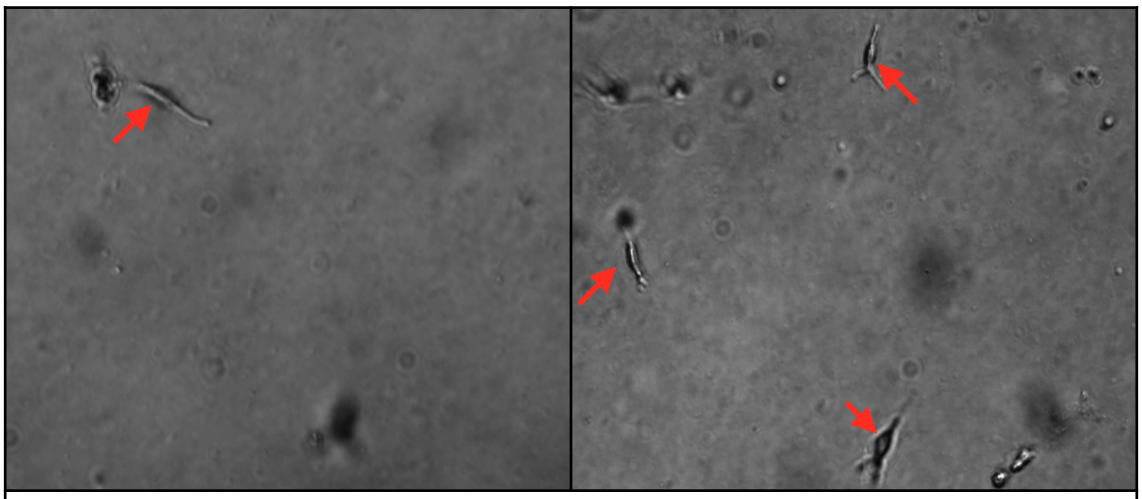
After a 24h incubation period, little angiogenesis activity could be appreciated in BAEC cultured in DMEM. On the case of VEGF-conditioned media, it could be clearly seen how the first cytoplasmic, filopodial, projections where appearing. The Kindler-conditoned BAECs, however, also exhibited some angiogenic activities, as was made manifest by the first cytoplasmic projections. These are not as pronounced as in the case of VEGF, but still great compared to the negative control, devoid of any neovascularization.

FIGURE 15: RESULTS OF BAEC CULTURE ON FIBRIN GEL AFTER 24H



NEGATIVE CONTROLS (10x)

POSITIVE CONTROLS (VEGF) (10x)

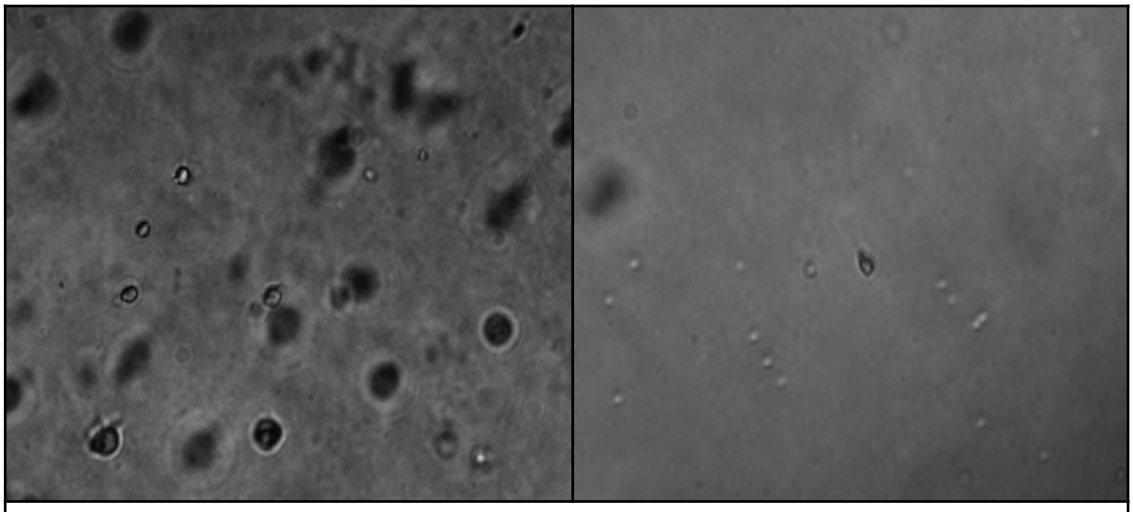


KINDLER (10x)

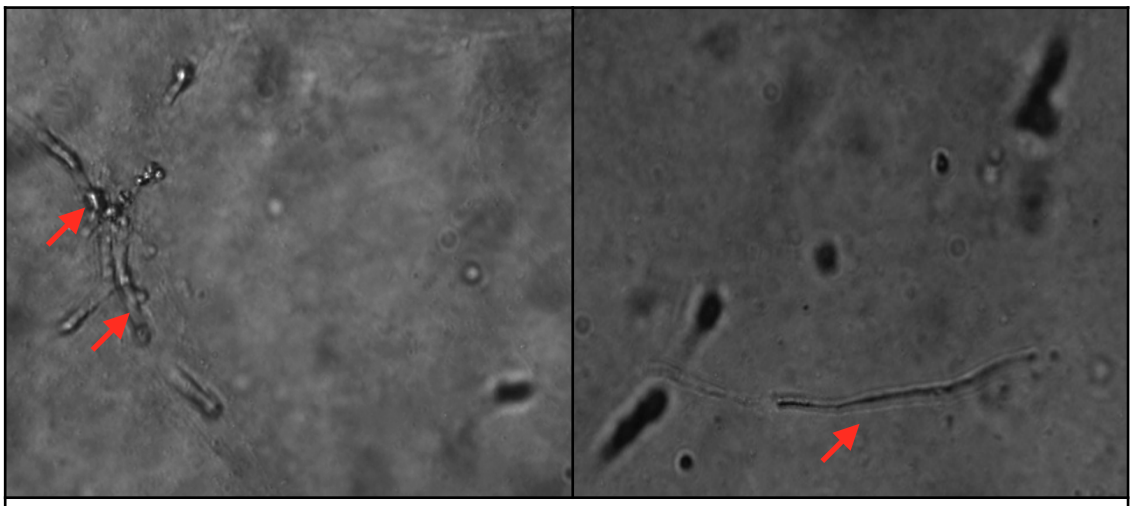
Results after 48h:

Another 24h hours later, 48h from the seeding of the cells, still no vasculogenesis could be observed in the negative controls. However, the filopodia in VEGF-conditioned BAECs could be observed to reach other isolated cells. Branching and elongation can be observed to begin. In the case of the Kindler-conditioned culture, a progression could also be observed. The cytoplasmic projections could be observed to develop and elongate, though mildly in comparison with the case of VEGF.

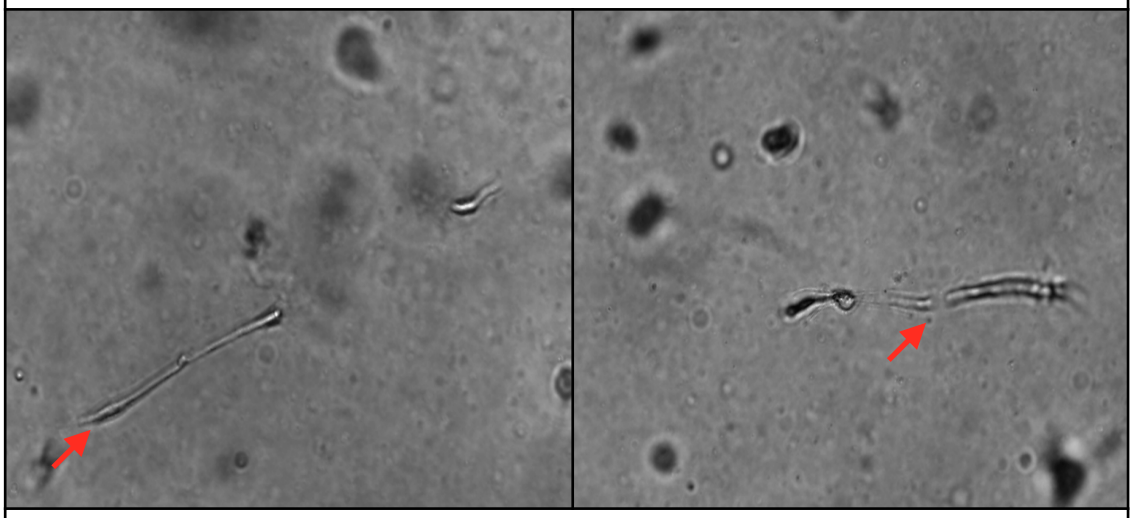
FIGURE 16: RESULTS OF BAEC CULTURE ON FIBRIN GEL AFTER 48H



NEGATIVE CONTROLS (10x)



POSITIVE CONTROLS (VEGF) (10x)



KINDLER (10x)

2. BAEC culture on Matrigel™

2.1. Culture on 1:1 Matrigel™ at a density 1,5•10^5 cells/well (Figure 16)

Microscopical observation of the cultures 1h after the time of media addition shows spontaneous cellular rearrangement into reticular-like disposition or web-like structures in all cultures except DMEM. After 24h, however, this reticular formations can no longer be observed.

24h after the addition of the conditioned media, still no signs of angiogenesis or vasculogenesis can appreciated in the DMEM negative control. However, the BAECs cultured conditioned by wild-type human keratinocytes

(HK-WT) do show angiogenic activities in the form of cytoplasmic fusion between neighboring cells (meshes) and the formation of tubular, branched structures resembling immature capillary vessels. As expected, BAEC cultured under VEGF conditions also exhibit these structures, which are similar in size and morphology to those found in the HK-WT wells. The Kindler-conditioned BAEC culture, also exhibits these formations.

After a 48h time lapse, some tubular formations could be observed on the DMEM culture, though in small numbers in comparison to the other cultures. The previously observed structures found on the WT-HK conditioned culture were observed to persist and even continue to grow and branch, enlarge and engross. So is the case of VEGF, although it is evident that the tubular structures formed have outgrown those found in any other well, both on length and caliber.

2.2. Culture on 1:2 Matrigel™ at a density of $2 \bullet 10^4$ cells/well (Figure 17)

Microscopical observation of the cultures 1h after the time of media addition shows no cellular rearrangement into reticular-like disposition like those observed for the previous configuration. On the VEGF and Kindler wells, however some highly branched elongations can be observed, formed by more than one cell.

24h after the addition of the conditioned media, little or no signs of either angiogenesis nor vasculogenesis can appreciated in the DMEM negative control. BAECs cultured with wild-type human keratinocytes (HK-WT) do show some tubular branched structures. However, BAEC cultured under VEGF conditions no longer exhibit structures like those found after 24h. In the case of HK-WT wells, little or no progression seems to have taken place over the last 24h either. Nor has the Kindler-conditioned BAEC culture.

After a 48h time lapse, no tubular structures could be found in any of the wells and there is no difference between the negative and positive controls. The kindler and HK conditioned cultures exhibit no difference from the negative control either.

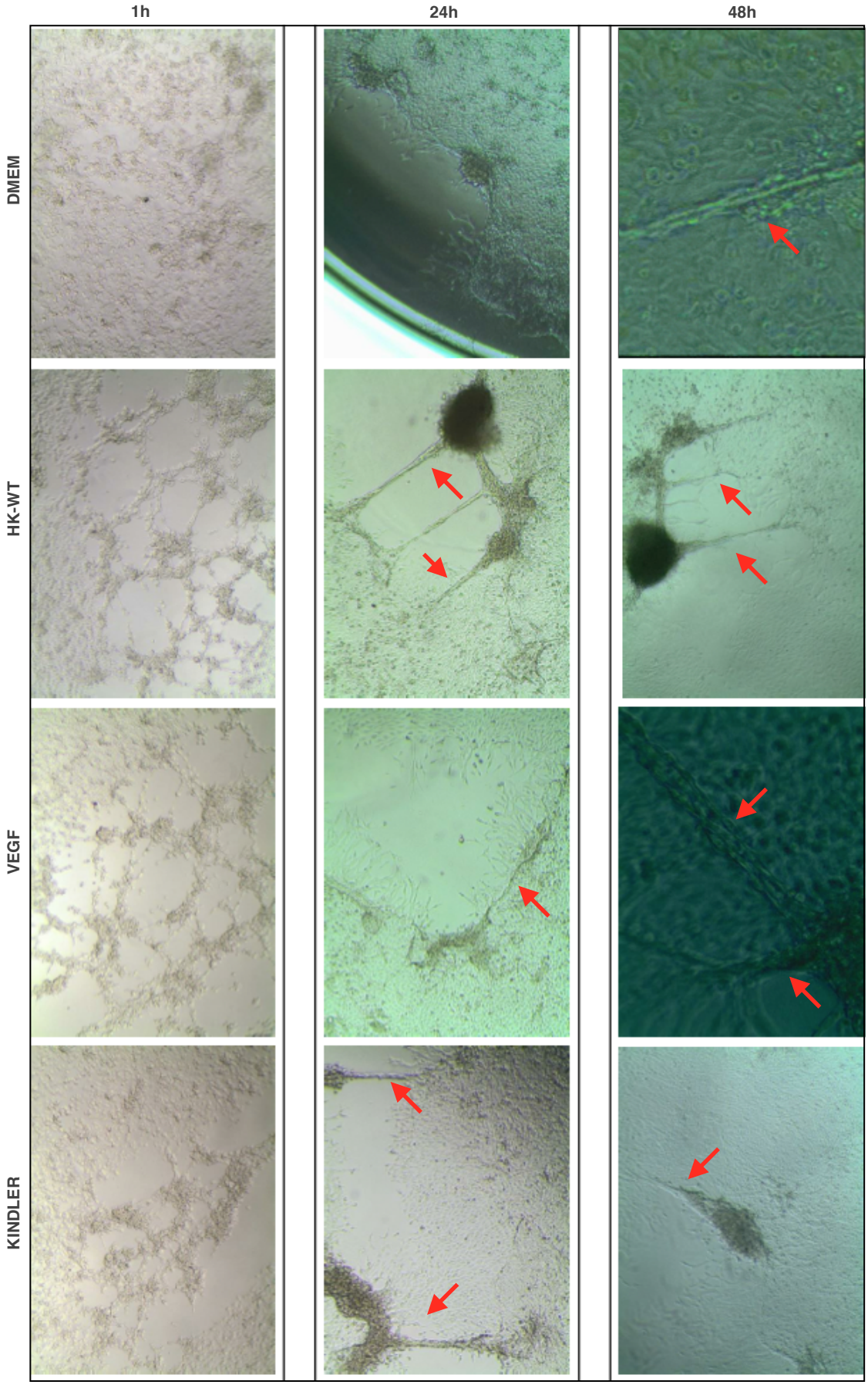


FIGURE 16: RESULTS OF BAEC CULTURE ON MATRIGEL™ 1:1 AT 1,5·10⁵ CELLS/WELL

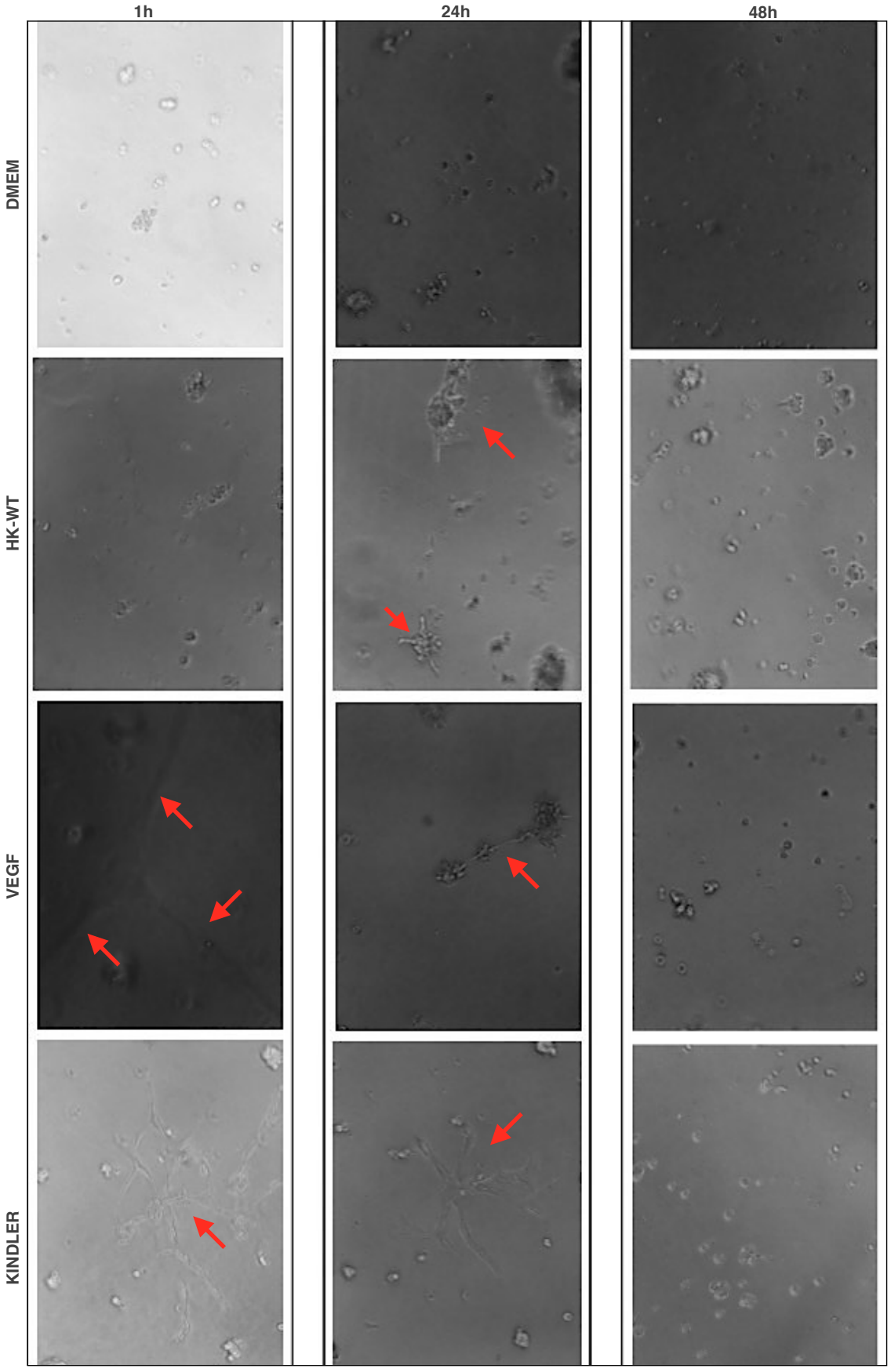


FIGURE 17: RESULTS OF BAEC CULTURE ON MATRIGEL™ 1:2 AT $2 \cdot 10^4$ CELLS/WELL

3. HUVEC culture on Matrigel™

Qualitative observation shows that 18 hours of conditioned media influence start to yield effect. In wells containing PEDF-conditioned media, the offset tubules produced in the 6th hour since the seeding at time 0h begin to recede. In the wells containing M199, tubules seem to decrease in caliber, forming meshes of bigger size than 18 hours before, though regression is also taking place.

FIGURE 18: RESULTS OF HUVEC CULTURE ON MATRIGEL™

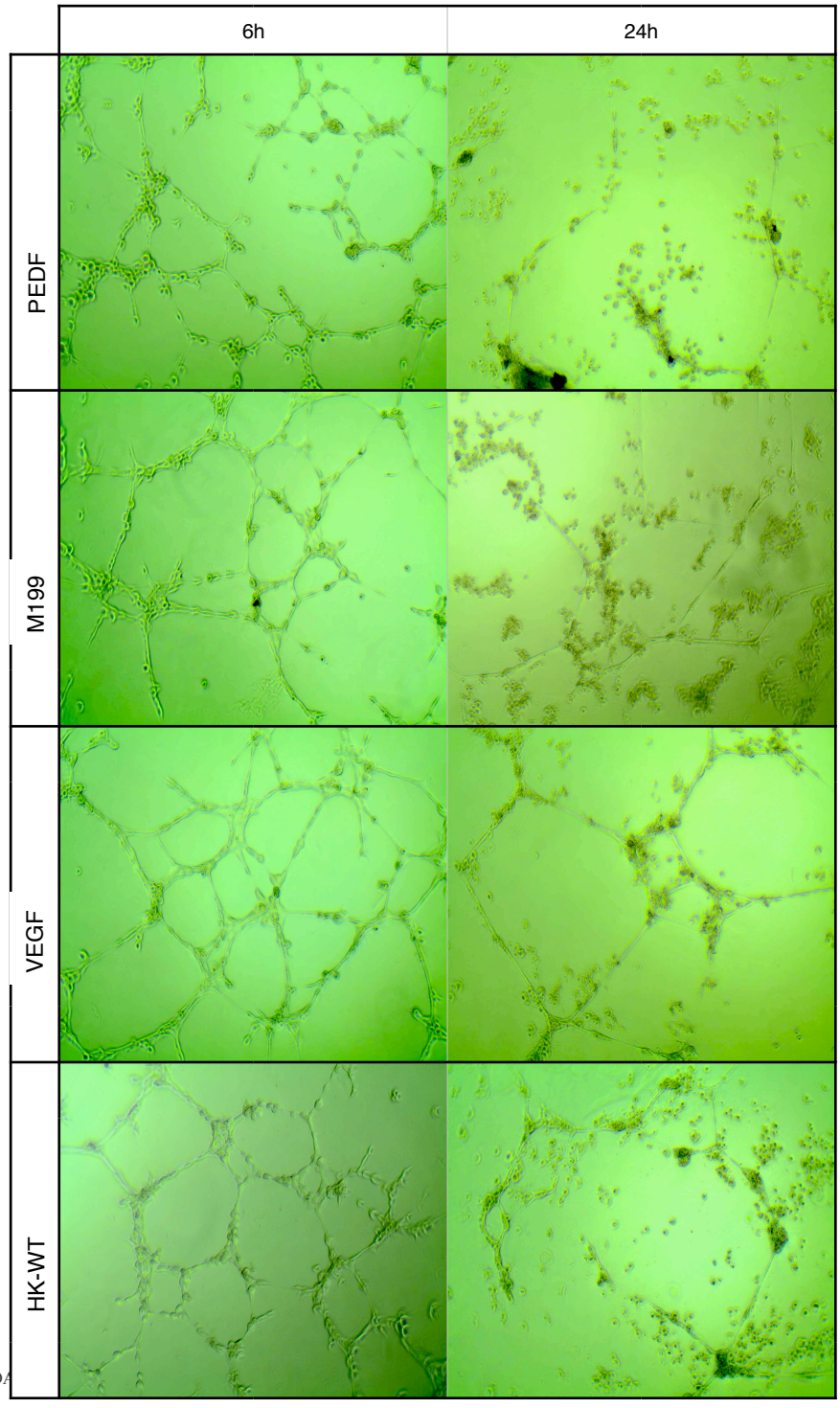
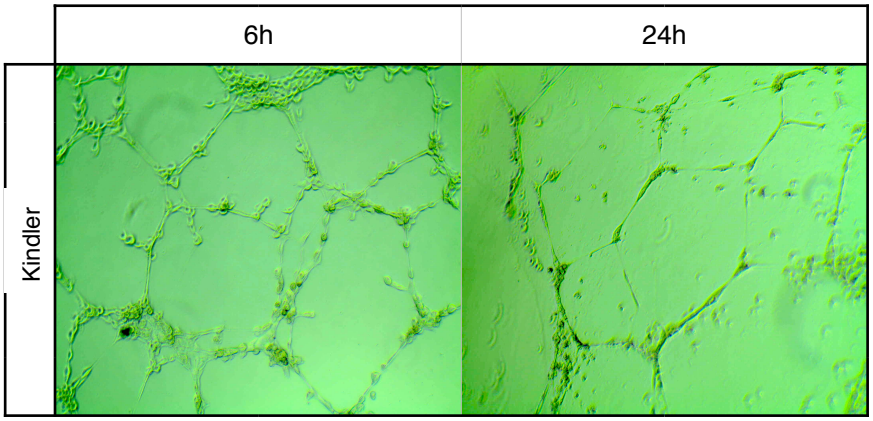


FIGURE 18: RESULTS OF HUVEC CULTURE ON MATRIGEL™

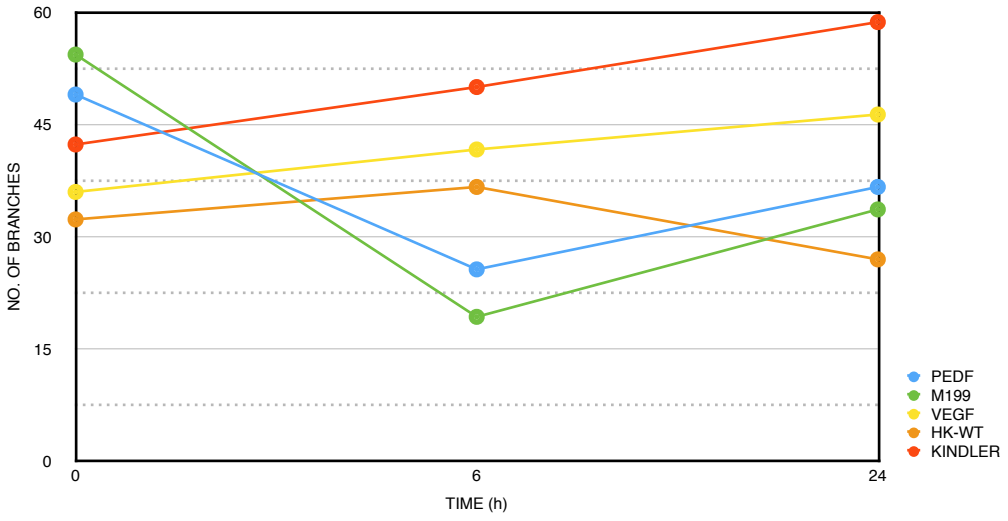


In the case of VEGF, the pre-existing network has developed into one of bigger meshes and longer tubes, contrary to those found in kindler-conditioned cultures, which haven't undergone regression but exhibit more and smaller meshes. (Figure 18)

A total of five parameters were measured in order to quantify both sprouting and intussusceptive angiogenesis. For the former, the number of branches and the total branch length where measured. Intussuceptive angiogenesis was quantified by counting the total number of meshes and measuring their mean mesh size and the total mesh surface area.

Six hours from the addition of conditioned media, the kindler culture exhibited the highest number of branches of all six conditions, followed by VEGF, keratinocyte medium and, least, by the PEDF and M199 cultures. 18 hours later, however, the PEDF culture followed by M199 exceeded the number of branches exhibited by that of the keratinocyte-conditioned culture. Kindler

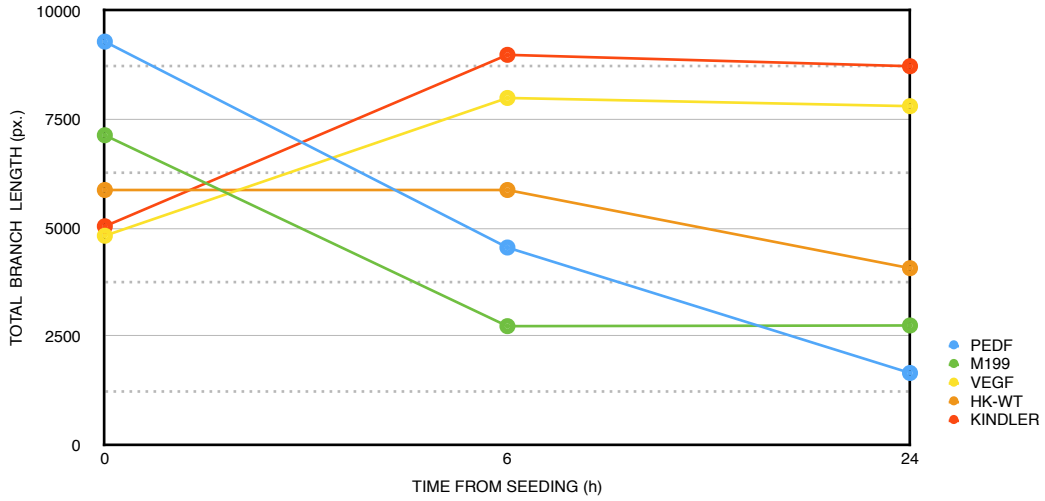
FIGURE 19: TIME Vs. TOTAL NO. OF BRANCHES



and VEGF after 24h, where still the cultures where the highest amount of new sprouted tubes could be found. (Figure 19)

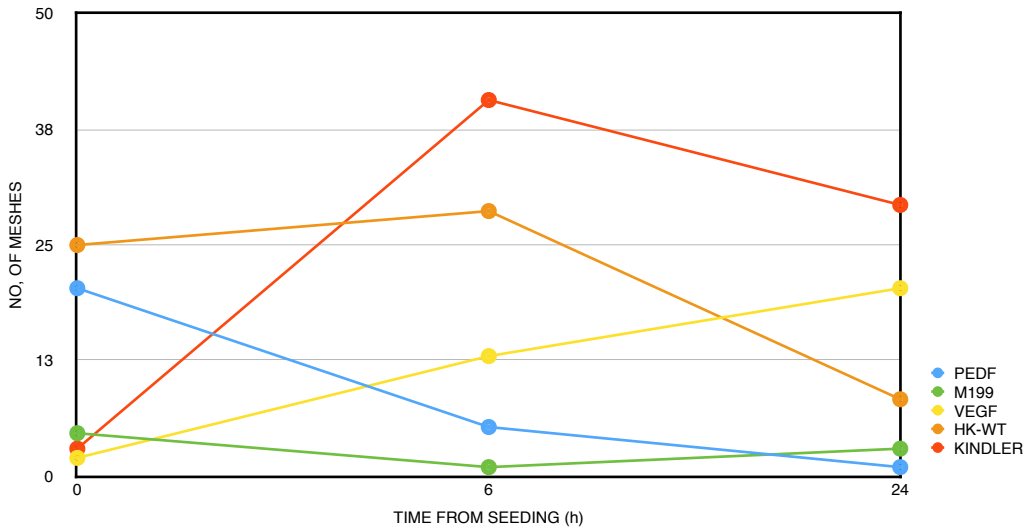
The total branch length, after 6h, was highest in the Kindler conditioned cultures. VEGF followed, and the shortest total branch lengths were found, respectively, in the PEDF and M199 cultures. These results were kept 18h later, 24h from the addition of the conditioned-media, except for M199 and PEDF, as the number of branches in the former now exceeded that of the latter. (Figure 20)

FIGURE 20: TIME Vs. TOTAL BRANCH LENGTH



The number of meshes, that is, the number of tubular formations characterized for the linkage of filopodia between different cells to form a network, was highest after 6h in the Kindler culture. The WT-keratinocyte conditioned culture presented the next highest number at this time, followed by VEGF, PEDF and M199. 18h later, the number of meshes in the Kindler culture

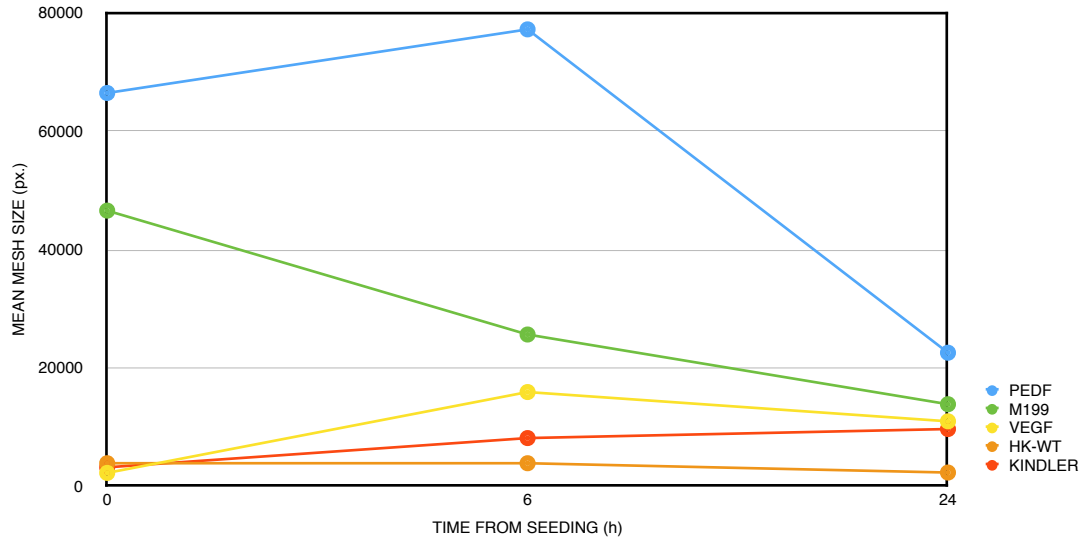
FIGURE 21: TIME Vs. NO. OF MESHES



was still the highest, though now followed by VEGF, which surpassed the WT-keratynocyte conditioned cultures. The number of meshes at this time became higher in M199 than in PEDF conditions. (Figure 21)

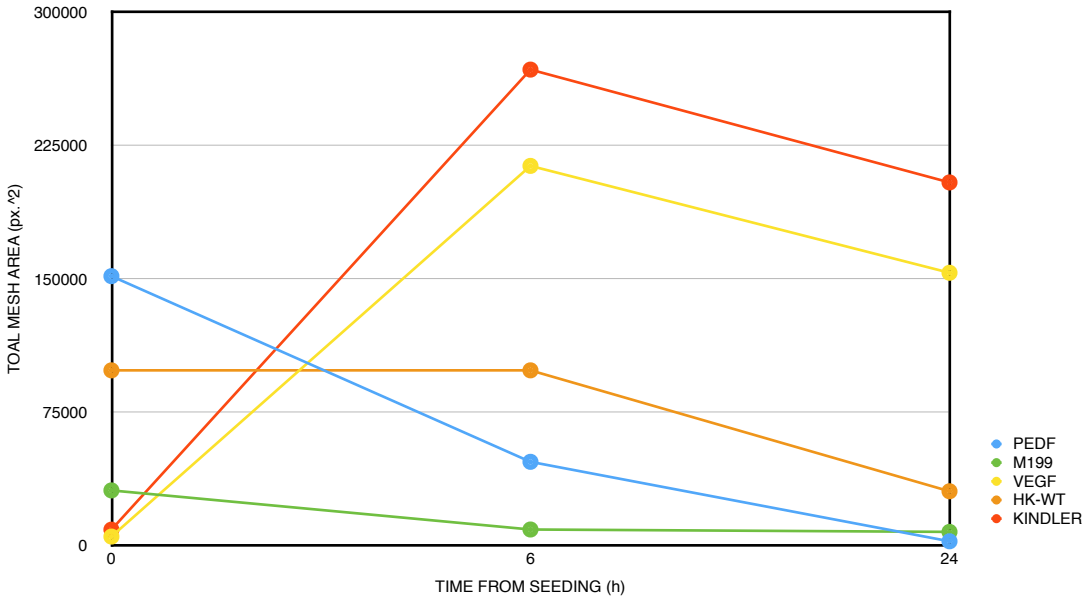
After 6h from the addition of the media, the mean mesh size was biggest in the PEDF-conditioned culture. Which exhibited almost twice as big meshes than the M199 culture, next in order of size. VEGF, Kindler and WT keratynocytes (in this order) exhibited the smallest meshes. Another 18h later, there was no such big difference between the different conditions as the mesh size is seen to regress in all groups. However, now PEDF exhibited the biggest mean mesh size, closely followed by M199, VEGF, WT-HK and, finally Kindler. It is worth mentioning that, whereas PEDF caused the mean mesh size to decrease over 24h, M199 and VEGF caused it to increase, while it was approximately maintained in for WT-HK. (Figure 22)

FIGURE 22: TIME Vs. MEAN MESH SIZE



The total mesh area, after 6h was highest in the kindler, closely followed by VEGF and almost doubling that next highest value, given by WT-HK. The value for the latter was kept approximately constant for the duration the 6h. PEDF follows, but the lowest value was given by M199. During the next 18h, the total mesh area decreases in all cultures and yet, by the 24th hour, the highest value is still found under Kindler conditions, followed by VEGF. These two groups almost double in mesh area the HK, M199 and PEDF cultures. The latter two will show almost no mesh area by the end of the 24th hour. (Figure 23)

FIGURE 23: TIME Vs. TOTAL MESH AREA



Discussion

The first experiment (M&M 1.1), that utilized BAECs cultured on fibrin gel was used as a preliminary study to determine a plausible proangiogenic effect of Kindler Syndrome (KS) and provided only qualitative insight of the premise. It could be observed that KS-conditioned medium from human keratinocytes did, indeed, unchain endothelial migration and rearrangement when compared to DMEM, not unlike VEGF-conditioned media. However, this first experiment had a main limitation, as it must be taken into account that the conditioned media were removed from genetically modified keratinocyte cultures. Thus, it could be argued that the observed angiogenic effects could be due to the effect of substances liberated by keratinocytes in normal conditions, independently of the the effect of the kindlin1 mutation.

The choice of the extracellular medium, or scaffold, for the first experiment was fibrin. It is characteristic of scar or fibrous tissue, which is formed when wound healing fails to regenerate the functional tissue. However, the scope of the study focuses on the dermo-epidermal junction of the skin, i.e. the basal lamina, where the effects of kindler are observed. Thus, though this scaffold provided a valid ECM for the culture of endothelial cells, it doesn't represent the physiological macromolecular milieu that is object of this study [44,45].

By culturing BAECs on MatrigelTM (M&M 1.2), the intention was to approximate the *in vitro* angiogenesis model to the physiological conditions of the body. BD MatrigelTM Basement Membrane Matrix is a solubilized basement membrane preparation rich in extracellular matrix proteins, including laminin (major component), collagen IV, heparan sulfate proteoglycans, and entactin/ nidogen. It is effective for the attachment and differentiation of epithelia and other cell types [53, 54]. BD MatrigelTM Matrix is best suited for applications where a more highly defined basement membrane preparation is desired. Hence this is the material of choice for this study where the pathology of KS is evaluated, since it is known to be involved in the anchorage of the actin cytoskeleton to integrin-associated signaling platforms [48].

It has been shown that tubule formation does not occur on tissues cultured without laminin or collagen IV [55]. If tubule formation is compared to

the previous experiment, where BAECs were seeded on a fibrin gel, it can be observed that not only wasn't there endothelial differentiation into capillary-like structures in the negative control but also the size and extent of the tubules obtained when using proangiogenic factors as VEGF were smaller and less abundant compared to Matrigel™. Accordingly, laminin is considered the major biologically-active component of the basement membrane in cellular organization and promotion of endothelial cell adhesion, migration, and differentiation [56]. This explains the HUVEC spontaneous arrangement into a vascular network on Matrigel™, which wasn't observed in the fibrin gel.

The interaction between WT-human keratinocytes was also interrogated in the second experiment (M&M 1.2). Results showed high angiogenic activity in this group, competing with that of the KS group and VEGF, supporting the thesis that the angiogenic activity produced by KS could be due to substances released by healthy keratinocytes [52]. In this aspect, the two configurations of experiment 1.2 were contradictory.

When seeding the cells at a density of $1{,}5 \cdot 10^5$ cells/well on 1:1 Matrigel™, the highest angiogenic activity was observed within the WT-HK wells, higher than in the VEGF and KS cases after 24h. These results aren't consistent with the fact that VEGF conditioned media from keratinocytes contains the same substances than HK-conditioned media, plus an excess of VEGF. In the case of 1:2 Matrigel™ seeded at $2 \cdot 10^4$ cells/well, the observed activity in the HK culture could be observed to be inferior to that of KS, which was highest, and to that of VEGF. This is more consistent, but all tubular formations where observed to recede another 24h later.

This experiment, however doubtful, did coincide with experiment 1.1 in the formation of tubular structures by BAEC cultured under KS conditions in comparison to DMEM. Therefore, the angiogenic relevance of Kindler Syndrome was once again manifested. The advantage of using Matrigel™ was made clear by the formation of meshed structures after 1h from seeding. This cellular arrangement, must be pursued in order to quantitatively evaluate angiogenesis similar to that occurring in physiological conditions. But in order to obtain stronger comparisons between groups, it was important to optimize the positive and negative controls. The introduction of WT-HK gave additional information concerning the VEGF and KS groups, but proved insufficient. Thus,

VEGF conditioned medium from modified keratinocytes will no longer be used. Besides, the negative control will be substituted by an angiogenesis inhibitor obtained from keratinocyte conditioned media: PEDF. This was expected to produce a reduced number of tubes when compared to DMEM in Matrigel™.

The introduction of PEDF and human recombinant VEGF protein was combined with a change in the type of cells used. For the third and last experiment (M&M 2), human primary endothelial cells were employed. Also, a narrower time frame of observation, 18h, was applied. The main improvement of this experiment with respect to the preceding ones, however, was in the form of a quantitative analysis using image segmentation software. This allowed to monitor two key parameters of sprouting angiogenesis and another three of intussusceptive angiogenesis. However, as the seeding of the HUVEC was done using Medium 199 before the conditioned media addition, angiogenesis was observed to initiate in every well as an offset. Thus, most plots show decreasing tendencies due to tube regression. The results obtained with the analysis software Angiogenesis Analyzer where also noisy due to the interference of high frequency elements such as debris and this interfered with the automated segmentation. However, images exhibiting this kind of interference were not included in the analysis to improve its accuracy.

Sprouting angiogenesis is, as previously stated, the process by which new vessels are formed by pre-existing ones. Mitogen gradients cause the projection of filopodia, which branch out and form a new vessel [26]. Thus, sprouting angiogenesis will be quantified by the measurement of the number of branches (unconnected tubes) and the total length of all branches. The results showed that KS produced the highest number of branches (Figure 2), more even than in the case of VEGF after 6 and 24h. This is consistent with results obtained in previous experiments and is indicative of the proangiogenic activity caused by keratinocytes expressing the KS genotype.

There are two aspects, however, which shed doubt upon these results. First, that PEDF produced more new branches than M199, which is the standard HUVEC culture medium, at hours 6 and 24; and second, that WT-HK produced the least amount of new branches. This last result contradicts those obtained in previous experiments, whereas the results for PEDF could be explained by the presence of other background substances produced by

keratinocytes. It must be noted that the conditioned media where provided without any information regarding their coposition, other than their origin.

The total length of all branches was highest on the KS and VEGF cultures which, again, is consistent with the results obtained in the previous experiments. As with the number of branches, after 6h, PEDF presented a higher total branch length than M199, though this changed after another 18h. Both in the number of branches and total branch length, ascending trends could be observed for KS and VEGF. This is not the case of PEDF, which exhibits a decreasing tendency. This is consistent with the previous experiments results and implies the creation of new sprouts in KS and VEGF, and sprout regression, or decreased proliferation and/or differentiation, in the case of DMEM and PEDF. This is supported by existing evidence, proving that skin wound conditions have the effect of enhanced blood vessel recruitment [38].

As it can be observed, HUVEC culture rapidly evolves into the formation of a reticular structure presenting meshes, which result from the merging of filopodia of neighboring cells. Intussusceptive angiogenesis *in vitro* is observed to occur when these meshes are divided into two by one, or more, intermediate filopodia [26]. Thus, the more meshes there are and the smaller, the higher the angiogenic activity. Analysis of the mesh number and total mesh area yield very similar results, indicating high angiogenic activity in KS, VEGF and HK cultures, specially in the KS culture when compared to HK. Whereas PEDF shows the lowest values, accompanied by M199. The anomalous results exhibited by M199 are, most probably, due to its age. Resulting in supplement inactivation.

When it comes to the mean mesh size, we would expect to find the smallest values in those cultures where more intussusceptive angiogenesis has taken place. As is the case of KS, VEGF and HK. This tendency, however, doesn’t contradict an increasing mesh area. As intussusceptive angiogenesis produces smaller, closed meshes, thus increasing the mesh number and total area. Of great importance is the result obtained here for HK, as the mean mesh size is found to remain constant, indicating little or no intussusceptive angiogenesis. This is consistent with the results obtained for HK in mesh number and area, which show slightly decreasing, almost constant trends. This result is supported by existing evidence on healthy keratinocytes’ vascularizing function, one of maintenance [38].

Conclusions

Microscopical observation of experiments 1.1, 1.2 and 2 strongly suggest a proangiogenic influence of keratinocytes expressing the Kindler genotype when compared to that DMEM and VEGF. The results obtained through the quantitative analyses of key factors such as the number of branches, the total branch length, the number of meshes, mean mesh size and total mesh area are consistent with these results.

Over a period of 18h, HUVEC cultured on artificial basement membrane Matrigel™ under KS conditions exhibited an increased number of branches and an increased branch length when compared to PEDF, the negative control, but also with respect to wild type human keratinocyte conditioned medium. This is indicative of an increased angiogenic activity in the form of sprouting angiogenesis. Also, the number of meshes and total mesh area measured in the KS wells were increased too when compared to those in the PEDF and HK cultures. This two parameters, in combination with the decreasing mean mesh size over a period of 18h in HUVECs cultured under KS conditions are indicative of an enhanced intussusceptive angiogenic activity. The obtained results and conclusions, though subject to noise and variability, are in consonance with known keratinocyte functions, which include the vascular support of the epidermis in healthy conditions and, more prominently, in disease and wound conditions such as those imposed by the Kindler Syndrome. The mutation on protein kindlin1 is causing the disruption of the cell signaling and anchorage machinery, resulting in the increased release of mitogens by keratonocytes.

Future Perspectives

In order to improve the results obtain and consolidate the conclusions, there are a series of improvements which could be performed on the previously described experimental set-ups. Matrigel™ should still be used for *in vitro* tube formation assays, as it is the adequate scaffold given the choice of cells and pathology. However, it would prove advantageous to remove the base medium before endothelial cell migration and differentiation happens, in order to reduce the initial offset. This also leads to the posterior regression of the tubes, increasing cellular debris and dead cells, which increases noise levels and leads to inconsistencies.

In order to reduce the noise levels, it would prove advantageous not to employ any conditioned media from keratinocytes except for KS and WT-HK, substituting PEDF conditioned media with human recombinant PEDF protein. On this aspect, using media from different patients would also increase the confidence of the conclusions. Also, the microscopical observation should be optimized to increase the field of view, in order to obtain more representative photographs. To this end, smaller magnification lenses should be used, or more photographs per well should be taken. Staining with calcein, or immunofluorescence targeting, would also help increase the accuracy of the automated segmentation, thus reducing noise.

Once the results obtained are consolidated by applying the aforementioned improvements. The best way to proceed would be to increase the complexity of the experiment to better recreate physiological tissue conditions. To this end, the co-culture of keratynocytes and endothelial (Mayumi Ono et al. 1992) would yield a more dynamic angiogenic induction. But explant, or whole tissue culture would also serve this purpose. The culture of murine aortic sections in Matrigel™ is a method of recognized validity for the measurement of sprouting angiogenesis (Nicosia et al 1990), though perhaps not the best recreation of the dermo-epidermal matrix. This last procedure was, in fact, explored before the HUVEC experiments but yielded not useful results.

Ex vivo validation of the results obtained in vitro would increase their validity, after which the identification of the messenger substances would be in order. For this purpose, a Western Blot, or any other relevant electrophoresis-

based analytical technique should be performed on the HK and Kindler-conditioned keratinocytes in order to identify the substances mediating the angiogenesis response. Once identified, in silica methods and data mining would yield a network of interactions and shed light into the underlying processes mediating the cross-talk between keratinocytes in healthy and diseased contains and endothelial rearrangement.

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ANNEX I: Expenditures

1. Reactants and cells:

Reactant	Manufact.:	Unit Cost (€)	Units Employed	*TOTAL COST (€)
Dulbecco's Modified Eagle's Medium (DMEM) - 500ml	Sigma-Aldrich	28,2	1	28,2
Matrigel 10ml	Corning bioscience	470,7	1	470,7
Recombinant Vascular Endothelial Growth Factor (VEGF) - 10μg	Sigma-Aldrich	302,5	1	302,5
Fetal Bovine Serum (FBS) - 500mL	Sigma-Aldrich	447,7	1	447,7
Collagenasa - 25mg	Sigma-Aldrich	37,10	1	37,1
Phosphate Buffered Saline (PBS) - 1L	Sigma-Aldrich	157	1	157
Medium 199 (M199) - 500ml	Sigma-Aldrich	22,7	1	22,7
Trypan® Blue-20mL	Sigma-Aldrich	9,8	1	9,8
Bovine aortic endothelial cells (BAEC) - 1 vial	Cell Systems	416,6	1	416,6
Human umbilical vein endothelial cells (HUVEC) - 1 vial	Cell Systems	321	1	321
			TOTAL EXPENSES	**2213,3**

2. Fungible Laboratory Glassware

Item	Units	*TOTAL COST (€)
T75 flasks	100	175,39
100mm petri dish	500	275
Flat bottom 96-well plate	50	54,4
1-10μl Pipette tips	1000	16,3
10-100μl Pipette tips	10000	30,94
100-1000μl Pipette tips	10000	30,94
15ml Falcon Flask	500	57,84
50ml Falcon Flask	500	50,78
10ml Pipettus tips	100	5,89
5ml Pipettus Tips	100	5,23
2ml Pipettus Tips	100	4,24
	TOTAL EXPENSES	**706,95**

3. Laboratory Hardware Inversion	
Item	**Unit Cost (€)**
Laminar Flow Cabinet	4500
Incubator	3699
Hot water bath	1084
Centrifuge	4545
1-10μl Pipette	150
10-100μl Pipette	150
100-1000μl Pipette	150
Pipettus	238
TOTAL EXPENSES	**14516**

4. Labour Costs (Laboratory)			
Operator	**Hours of Work**	**Cost of hour (€)**	**TOTAL COST (€)**
Biomedical Engineering/Other	300	15	**4500**

TOTAL COSTS	
Reactants and cells	2213,3
Fungible laboratory glassware	706,95
Laboratory hardware	14516
Labour costs	4500
TOTAL EXPENSES	**21936,25**

* Costs don't include associated taxes (VAT)

Acknowledgments

This work wouldn’t have been possible without the collaboration of fellow student Javier Cudeiro Blanco. With whom the BAEC experiments where performed and planned as part of the courseworks of *Cell Culture and Biotechnology for Tissue Engineering Advanced* and *Biomaterials Experimental Design,* which provided the preliminary evidences.

Special thanks are due for the research team lead by Dr. Blanca Duarte, from Centro de Investigaciones Energéticas y Medioambientales (CIEMAT), as without their cells (BAEC and HUVEC) and conditioned media the experiments couldn't have been performed.

Last, and most important, many special thanks to Dr. Adela García Martín and Dr. J. Claudio Conti, and to the members of the bioengineering tech team: Angélica Corral, Miguel Íñigo Parmaann and Guillermo Vizcaíno Navas. Without their patience (specially Dr. García Martín’s), their constant help and guidance none of this would have been possible.